UNIVERSITÉ DE MONTRÉAL

EFFECT OF INTERFERON-TAU PROTEIN SECRETION IN BOVINE ENDOMETRIAL CELLS AND ITS MODULATION BY STEROID HORMONES

par

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Thèse présentée à la Faculté des études supérieures
En vue de l'obtention du grade de
Philosophiae Doctor (Ph.D)
en sciences vétérinaires
option reproduction

Octobre, 2004

UNIVERSITÉ DE MONTRÉAL
FACULTÉ DES ÉTUDES SUPÉRIEURES

Cette thèse intitulée :

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Dès le début de la gestation chez les ruminants, l'embryon n'a pas comme seul rôle de prévenir la sécrétion de la prostaglandine $F_{2\alpha}$ mais module également la sécrétion des protéines endométriales. L'interféron-τ (IFN-τ) est produit par le trophoblaste avant l'implantation chez les ruminants. Ce fateur est impliqué dans la reconnaissance maternelle de la gestation, et peut altérer la synthèse de protéines endométriales et inhiber la prolifération de certaines cellules, étant une molécule pléïotropique. Toutefois, les mécanismes impliqués dans la reconnaissance maternelle de la gestation, et dans l'implantation et le développement embryonnaires ne sont pas bien connus aux niveaux cellulaire et moléculaire. Pour améliorer notre compréhension , un système de culture primaire de cellules endométriales a été utilisé dans la présente étude. Nous proposons comme **hypothèse** que IFN-τ peut modifier la sécrétion des protéines endométriales qui sont impliquées dans l'établissement de la gestation. Les **Objectifs** de cette étude d'étaient d'établir un système de culture de cellules endométriales bovines pouvant répondre aux hormones stéroïdiennes et IFN-τ, puis d'examiner les effets de IFN-τ sur la sécrétion de protéines endométriales, et la modulation possible de cette sécrétion par les hormones stéroïdiennes.

Dans la première expérience, l'effet de IFN-τ sur la sécrétion de protéines par les cellules épithéliales ou stromales de l'endomètre bovin a été examiné par gel SDS PAGE bi-dimensionnel (2D SDS-PAGE) et par analyse HPLC. Les résultats ont démontrés que IFN-τ induit la sécrétion de plusieurs protéines par les cellules épithéliales de l'enomètre bovin et ces effets sont modulé par les hormones stéroïdiennes. L'estradiol régule négativement la sécrétion de protéines, tandis que la

progestérone régule la sécrétion de protéines positivement. L'analyse subséquente des séquences protéiques a montré que ces protéines contenaient des séquences partielles d'acides aminés correspondant au facteur inhibiteur de migration des macrophages (MIF).

Dans la deuxième expérience, l'expression de MIF dans les cellules épithéliales de l'endomètre bovin a été examinée par buvardage de type Nothern, de type Western et par analyse immunohistochimique. La sécrétion de MIF par les cellules épithéliales bovines a été évaluée par buvardage de type Western. Les résultats ont démontrés qu'une grande expression basale de la protéine et de l'ARN messager (ARNm) de MIF était observée dans toutes les cellules, mais que les traitements hormonaux et les contrôles n'ont pas d'effet sur cette expression. Toutefois, IFN-τ stimule la sécrétion de la protéine MIF par les cellules. La caractérisation de la régulation de l'expression de la protéine MIF et de son ARNm par IFN-t contribuerait à améliorer de façon significative notre compréhension des intéractions embryo-utérines du début de la gestion chez les ruminants.

Pour la troisième expérience, l'effet de IFN-τ sur l'apoptose dans les cellules épithéliales de l'endomètre bovin en culture a été examiné par analyse TUNEL, par fragmentation d'ADN, et par buvardage de type Western. Les résultats indiquent que IFN-τ et CHX augmente significativement le pourcentage de cellules à noyau apoptotique (34.3 et 46.5% respectivement), comparativement au contrôle (11.2%) ($P<0.05$). Le traitement des cellules avec la progestérone inhibe significativement l'habileté de IFN-τ à induire l'apoptose (13.7%), comparativement à IFN-τ seul (34.3%) ($P<0.05$). L'analyse de fragmentation d'ADN a démontré que le traitement avec INF-τ

résulte en une augmentation de la séparation de l'ADN relativement aux cultures contrôles non-traitées. Le buvardage de type Western a démontré que les traitements avec IFN-τ et la cycloheximide résultent en une augmentation d'expression de la protéine pré-apoptotique Bax, comparativement aux cultures contrôles.

En **conclusion**, ces données expérimentales démontrent que IFN-τ est capable d'induire la sécrétion de protéines par les cellules épithéliales de l'endomètre bovin, de réguler positivement la sécrétion de MIF, et d'induire l'apoptose dans ces cellules. De plus, ces effets sont modulés par les hormones stéroïdiennes. La présente étude est d'importance car elle démontre pour la première fois que (1) l'ARNm et la protéine MIF sont hautement exprimés dans les cellules épithéliales de l'endomètre bovin en culture, et que la sécrétion de MIF est stimulée en réponse IFN-τ; (2) IFN-τ inhibe la prolifération des cellules épithéliales et induit l'apoptose dans les cellules épithéliales de l'endomètre bovin en culture. L'implication de IFN-τ dans la reconnaissance maternelle de la gestation est connue depuis plusieurs années, mais l'étude présente rapporte pour la première fois l'implication directe de cette cytokine dans l'induction de l'expression d'un membre de la famille bcl2, Bax-α, et de l'apoptose dans les cellules épithéliales de l'endomètre bovin. Une autre découverte importante est que l'apoptose induite par IFN-τ est inhibée par la progestérone, ce qui suggère que les concentrations plasmatiques de progestérone au début de la gestation sont importantes pour la modulation des effets de IFN-τ.

Ces résultats rassemblés suggèrent que MIF est vraisemblablement un facteur contribuant à l'établissement du début de la gestation, mais la signification fonctionnelle de MIF reste à être déterminée. Comprendre la régulation de la sécrétion de MIF et son

site d'action dans le tractus reproducteur ajoutera significativement à notre compréhension des intéractions embryo-utérines du début de la gestation. Nous spéculons que IFN-τ pourrait jouer un rôle dans le développement de l'endomètre autour de la période d'implantation. Incidemment, la compréhension de la régulation de la sécrétion de la protéine MIF par IFN-τ ajoutera significativement à notre compréhension des intéractions embryo-utérines du début de la gestation.

Summary

During early pregnancy in ruminants, the embryo not only prevents prostaglandin $F_{2\alpha}$ release, but it also modifies protein secretion from the endometrium. Interferon-t (IFN-t) is produced by the trophoblast prior to implantation in ruminants. It is involved in maternal recognition of pregnancy and is also a pleiotropic molecule that can alter the synthesis of endometrial proteins and inhibit proliferation of some cells. However, the mechanisms involved in maternal recognition of pregnancy, embryo implantation and development are not well understood at the cellular and molecular level. To further our understanding, a primary culture system of endometrial cells was used in these studies. We **hypothesize** that IFN-τ modifies protein secretion from endometrium that is involved in the establishment of pregnancy. The **objectives** of this study were to establish an appropriate primary endometrial cell culture system that can respond to steroid hormones and IFN-τ and to examine the effect of IFN-τ on protein secretion and cell division and the possible modulation of its effects by steroid hormones.

In the first experiment, the effect of IFN-τ on protein secretion from bovine endometrial epithelial cells and stromal cells was examined by 2-D SDS PAGE and HPLC analysis. The results showed that IFN-τ induced the secretion of several proteins from bovine uterine epithelial cells and this was modulated by steroid hormones. Estradiol down-regulated protein secretion and progesterone up-regulated protein secretion. Protein sequence analysis of proteins induced by IFN-τ showed partial amino acid sequences that corresponded to macrophage migration inhibitory factor (MIF).

In the second experiment, expression of MIF in isolated epithelial and stromal cells of bovine endometrium was examined by Northern blotting, Western blotting and immunohistochemistry analysis. MIF secretion from cultured bovine epithelial cells was examined by Western blotting analysis. Results showed that MIF protein and MIF mRNA were expressed in epithelial, but not in stromal cells. There was no effect of IFN-τ on MIF expression in the epithelial cells. However IFN-τ did stimulate the secretion of MIF protein from the cells. The characterization and understanding of the MIF protein and mRNA expression and regulation of the secretion of the MIF protein by IFN-τ will add significantly to our understanding of early embryo-uterine interactions.

In the third experiment, the effect of IFN-τ on cell growth was studied. Results showed that IFN-τ at the 100 ng/ml dose, either alone or in the presense of P4, significantly decreased the DNA content of epithelial cells ($p < 0.001$), indicating that a decrease in proliferation or an increase in cell death, or both, occurred. To further examine the reason for this effect, apoptosis in cultured epithelial cells of bovine endometrium was examined by TUNEL, DNA fragmentation and western blotting analysis. The results showed that IFN-τ and CHX significantly increased the percentage of cells with apoptotic nuclei (34.3 and 46.5% respectively) when compared with control (11.2%) ($P<0.05$). Progesterone treatment of the cells significantly inhibited the ability of IFN-τ to induce apoptosis (13.7%) when compared with IFN-τ alone (34.3%) ($P<0.05$). DNA fragmentation analysis showed that INF-τ treatment resulted in an increase in the appearance of DNA laddering compared with that in untreated control cultures. Western blotting analysis showed that IFN-τ and cycloheximide treatment

resulted in an increase in the expression of the proapoptotic protein Bax-α compared with that in control cultures.

In conclusion, these data demonstrate that IFN-τ can induce protein secretion from epithelial cells of bovine endometrium, stimulate MIF secretion and induce apoptosis in bovine uterine epithelial cells. These effects were modulated by steroid hormones. The most significant findings in those studies are that: (1) it demonstrates for the first time that MIF mRNA and protein are highly expressed in cultured bovine endometrial epithelial cells and that the secretion of MIF is stimulated in response to IFN-τ. (2) IFN-τ inhibits epithelial cell proliferation and induces apoptosis in cultured bovine endometrial epithelial cells. Although the involvement of IFN-τ in maternal recognition of pregnancy has been known for a number of years, this is the first report that this cytokine directly induces a bcl2 gene family member, Bax-α, and apoptosis in bovine endometrial epithelial cells. The other important finding is that the IFN-τ-induced apoptosis is inhibited by progesterone, which suggests that plasma progesterone concentration during early pregnancy is important for modulating the effect of IFN-τ. Taken together, our results suggest that MIF is likely a factor contributing to the establishment of early pregnancy, however, the functional significance of MIF remains to be determined. Understanding the regulation of MIF secretion and its site of action in the reproductive tract will add significantly to our understanding of early embryo-uterine interactions.

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LIST OF ABBREVIATIONS

ACTH	Adrenocortropic hormone
BGCP-2	Bovine granulocyte chemotactic protein-2
bIFN-τ	Bovine interferon-τ
BSA	Bovine serum albumin
bTP-1	Bovine trophoblast protein-1
CL	Corpus luteum
COX	Cyclooxygenase
CTB	Inner cytotrophoblast
CHMI	5-Carboxymethyl-2-Hydroxymuconate isomerase
DEPC	Diethyl Pyrocarbonate
DR-6	Death receptor-6
DTH	Delayed-type hypersensitivity
E2	Estradiol-17β
EGF	Epidermal growth factor
ER	Estradiol receptor
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBSS	Hank's buffered saline solution
HCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor

HPA	Hypothalamus-pituitary-adrenal axis
IgG	Immunoglobulin G
IGF-2	Insulin-like growth factor-2
IFN-τ	Interferon-τ
IL-1	Interleukin-1
IRE	Interferon response element
IRF	Interferon regulatory factor
ISGF-3	IFN-stimulated gene factor-3
LPS	Lipopolysaccharide
NBCS	New-born calf serum
MIF	Macrophage migration inhibitory factor
NO	Nitric oxide
NOS	Nitric oxide synthase
ODF	Outer dense fibres
OT	Oxytocin
oTP-1	Ovine trophoblast protein-1
OTR	Oxytocin receptor
P_4	Progesterone
$PGF_{-2\alpha}$	Prostaglandin $F_{-2\alpha}$
PGFS	Prostaglandin F synthase
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
PGHS	Prostablandin G/H synthase

PR	Progesterone receptor
RA	Rheumatoid arthritis
rbIFN-τ	Recombinant bovine interferon-τ
SDS	Sodium dodecyl sulfate
STB	Outer syncytiotrophoblast
TGFα	Transforming growth factor-α
TIMPs	Tissue inhibitors of metalloproteinases
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP nick end-labelling technique
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNFR family and through binding to its receptor
TRADD	TNFR1-associated death domain
TSST-1	Toxic shock syndrome toxin-1
UCRP	Uniquitin cross-reactive protein
VSV	Vesicular stomatis virus

Acknowledgements

First of all I would like to sincerely thank my supervisor Dr Alan K. Goff for his invaluable guidance, maximum patience, and constant encouragement, unselfish help and very thoughtful arrangement for my study while I was studying at the Centre de recherche en reproduction animale (CRRA), University of Montreal. In addition, there are many people I need to thank for their help in the completion of this thesis and my Ph.D. studies. I am greatly indebted to the members of my graduate student advisory committee, Drs Bruce D. Murphy, Christopher A. Price and Denis Vaillancourt.

I also would like to thank Dr R. M. Roberts for his generous gift of the recombinant bovine interferon-τ and Dr A. Meinhardt for his MIF antibody and probe which allowed me to begin the study of MIF expression in bovine endometrial epithelial cells without delay.

This study was carried out at CRRA, Faculté de médecine vétérinaire. I would like to thank all members of the CRRA, for their friendship and kindness during the course of my study. Particularly, I am grateful to Mrs Dobias-Goff, Daniell Rannou for their technical assistance. I am grateful to Miss Joëlle Desmarais for her help in French translation of my thesis summary. I am also grateful to Drs Derek Boerboom, Angelika Stock, Khampoune Sayasith, Nicolas Gévry and Mingju Cao not only for their help invaluable for the completion of this work but also for their friendship.

I am indebted to express my sincere thanks to my wife Wenya, who supported and encouraged me in spirits, to my son Kaiyang and daughters Anna and Gina for their love. Finally, I am indebted to my parents-in-law and my mother for their encouragement in spirits and taking care of my lovely children during the period of my study.

AVANT-PROPOS (PREFACE)

Cette thése comprend une introduction générale qui renferme l'hypothése de départ, une revue de littérature générale; trois articles comprenant chacun une introduction, une section Matériel et méthodes, des résultats, une discussion et des références; une discussion générale ainsi qu'une conclusion générale.

This thesis composes a general introduction which indicates the hypothesis of the study, a general literature review; three articles, each of which contains specific introduction, materials and methods, results, discussion and references; a general discussion and general conclusion.

1. 0. Introduction

Maternal recognition of pregnancy results from biochemical signaling between the conceptus (embryo and its associated membrances) and the maternal system. Pregnancy recognition signals ensure maintenance of structural and functional integrity of the corpus luteum (CL), which would otherwise regress at the end of the estrous cycle. The CL produces progesterone, the hormone of pregnancy, which is responsible for maintaining endometrial functions that permit early embryonic development, implantation, placentation, and successful fetal/placental development.

The estrous cycle of cows is uterine dependent, and the luteolytic signal responsible for structural and functional demise of the CL, or luteolysis, is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The uterine endometrium, primarily luminal epithelium and perhaps superficial glandular epithelium, is influenced by progesterone, estrogen, and oxytocin, through their cognate receptors, to release pulses of $PGF_{2\alpha}$ required for luteolysis. The antiluteolytic signal for pregnancy recognition in cows is a novel Type I interferon named interferon-τ (IFN-τ)(Roberts, Farin et al. 1990). IFN-τ is secreted by trophectoderm of cow conceptus between Day 12 and Day 25 of pregnancy and exerts a paracrine effect on the uterine endometrium to abrogate the luteolytic mechanism (Mann and Lamming 2001).

Pregnancy starts with fertilization and ends with parturition (the birth process). After fertilization, the conceptus develops through periods of cleavage, differentiation, and growth. Until differentiation is completed, the conceptus is

called an embryo, after differentiation, it is called a fetus. During the early part of gestation, the embryo remains free, first in the oviduct and then the uterus. The establishment and maintenance of pregnancy involves a series of molecular signals exchanged by conceptus (embryo/foetus and associated membranes) and endometrium. During the peri-implantation period the ruminant conceptus is bathed in endometrial secretions (histotroph) which nourish and sustain it. Histotroph contains a complex mixture of proteins, carbohydrates, sugars, lipids and ions (Bazer 1989). Changes in the quantitative and qualitative pattern of endometrial protein in cyclic and pregnant ruminants have provided clues to the identity and function of the protein components of histotroph and their roles in establishment of pregnancy .

During early pregnancy the uterus, under the influence of ovarian steroids progesterone and estrogen, undergoes cellular and molecular changes to achieve a receptive state for the onset of implantation. In rats and mice, progesterone and estrogen sequentially program the uterus into pre-receptive, receptive and non-receptive phases during pregnancy or pseudopregnancy (Psychoyos 1973). Blastocysts implant only in the receptive uterus. The progesterone-primed pregnant uterus becomes receptive on day 4 after it is superimposed with preimplantation ovarian estrogen secretion. Subsequently, the receptive uterus proceeds to a non-receptive phase when blastocysts can no longer implant. Although many molecular signaling pathways have been identified associated with uterine receptivity and embryo–uterine interactions during implantation, the

definitive roles of these pathways or their interactions in these processes remain elusive.

In this respect, scientists have recently discovered that the mouse uterus, which was thought to be receptive primarily on day 4, is still receptive on day 5 of pseudopregnancy after blastocyst transfers. For example, mice receiving blastocyst transfers on day 5 of pseudopregnancy show implantation when examined 48 h later. In contrast, similarly transferred blastocysts completely failed to implant in day 6 pseudopregnant recipient uteri (Song, Guan et al. 2002). Before implantation (Day 21) the embryo depends on uterine secretion for normal development and survival. Only after placentation, can the embryo derive nutrients and transfer waste products through maternal blood. In the cow between 30 and 35 days after fertilization, there will be 3 or 4 fragile cotyledonary attachments in the pregnant horn (Song, Guan et al. 2002).

Cytokines play a pivotal role in regulating the host inflammatory and immune responses to infection and tissue invasion. They regulate the first non-specific phase of the host response by orchestrating a local inflammatory reaction and then serve to control the subsequent specific immune response. Cytokines appear to play a critical role in the establishment of pregnancy. MIF is a pro-inflammatory cytokine involved in reproduction (Ietta, Todros et al. 2002).

During pregnancy, the uterus and the placenta are immunologically privileged sites in which immune activity is effectively diminished (Streilein 1995). Apoptosis of immune cells has been proposed as a mechanism for

maintaining immune privilege (Griffith and Ferguson 1997). Induction of apoptotic cell death can also be a factor that limits lymphocyte proliferation following activation. Apoptosis is a complex process that removes aging or injured cells from the body and occurs in a wide variety of organisms. Regulation of apoptosis is complex and involves a family of related proteins that can promote or inhibit this process. Apoptosis may serve as a previously unsuspected mechanism that induces tolerance of the foetal allograft against maternal immune system and plays a role in early pregnancy maintenance (Jerzak and Bischof 2002).

Based on the above considerations, we hypothesize that IFN-τ modifies protein secretion from endometrium that are involved in the establishment of pregnancy. The specific objectives of this project were (1) to establish an in vitro model to explore the effect of IFN-τ on protein secretion from cultured bovine endometrial epithelial and stromal cells, (2) to identify proteins secreted into culture medium and effected by IFN-τ in bovine endometrial epithelial cells, and (3) to examine its steroid hormone modulation on protein secretion induced by IFN-τ.

2. 0. LITERATURE REVIEW

This literature review will focus on (1) the events that occur during early gestation (the first three weeks) in the cow, including the hormonal influences on the outcome of early pregnancy, production of IFN-τ (embryo signal), and the luteolytic mechanism (maternal signal) induced by $PGF_{2\alpha}$, uterine hormone

receptors during early pregnancy, uterine remodelling and protein synthesis, cleavage, implantation and embryo development; (2) role of MIF in inflammatory and reproduction and highlight the functional and mechanistic properties of MIF, and (3) the role of apoptosis in early pregnancy maintenance.

2.1. Maternal Recognition of Pregnancy

2.1.1 Hormonal Influences on the Outcome of Early Pregnancy

Many of the mechanisms involved in early pregnancy are influenced by the ovarian steroid hormones, progesterone and estradiol, and many studies have investigated their roles. It has been established for many years that the concentration of progesterone during early pregnancy has a marked effect on the potential outcome. Lower concentrations of plasma progesterone from about day 12 after mating have been reported in animals in which early pregnancy fails in a number of studies (Lukaszewska and Hansel 1980; Lamming, Darwash et al. 1989; Mann and Lamming 1995). These studies clearly demonstrate that both a late post-ovulatory rise in progesterone and low luteal phase concentrations of progesterone have a detrimental effect on the outcome of early pregnancy.

Although estradiol concentrations have not been studied as comprehensively as concentrations of progesterone during early pregnancy, most studies indicate that concentrations of estradiol do not differ between mated cows in which pregnancy is successful or fails (Lukaszewska and Hansel 1980; Gyawu and Pope 1992; Mann and Lamming 1995). In one study in beef cows, a lower pregnancy rate was observed in cows with higher plasma concentrations of

estradiol between day 14 and day 17. However, in this study, luteolysis had begun in some cows and so it is not clear whether the higher concentration of estradiol was the cause of pregnancy failure or the result of failed embryonic inhibition of luteolysis (Pritchard, Schrich et al. 1994). Thus current evidence supports the idea that estradiol does not exert the same degree of influence as progesterone over the outcome of early pregnancy. It has now been demonstrated that the concentration of luteal phase progesterone in the cow has a profound influence on the strength of development of the luteolytic signal (Mann and Lamming 1995). Estradiol concentrations do not differ between pregnant and non-pregnant cows, but have an important influence in controlling the strength of the luteolytic signal (Mann and Lamming 1995).

2.1.2. The Embryonic signal--Production of Interferon-τ

In ruminants, IFN-τ is well characterized as an important embryonic pregnancy recognition signal. IFN-τ is encoded by multiple genes (Ealy, Larson et al. 2001) and is expressed and secreted by trophectoderm cells of blastocysts (Roberts, Farin et al. 1990). Secretion of IFN-τ by bovine blastocysts *in vivo* is highest between days 15 and 17, whereas in a specific culture system for bovine trophoblastic vesicles increasing IFN-τ secretion was observed for a longer period (up to day 23 after fertilization) (Stojkovic, Wolf et al. 1995).

In cows, removal of the embryo from the uterus on day 15 does not result in a delay in luteolysis, whereas removal on day 17 results in a significant delay. Furthermore, infusion of homogenates of day 17-18 embryos resulted in a delay

in luteolysis (Northey and French 1980). Thus it was established that the embryo exerted an anti-luteolytic effect on the cow between day 15 and day 17. Highly purified bTP-1 obtained from culture of day 17-18 conceptuses (Helmer, Hansen et al. 1989) and recombinant bovine interferon-τ (Meyer, Hansen et al. 1995) have been shown to reduce luteolytic secretion of $PGF_{2\alpha}$ and extend luteal function in the cow. Expression of mRNA for interferon-τ has been detected as early as day 12 in the cow, is maximal on days 15-16 and continues at least until day 25. This expression appears to be limited to the trophectoderm and expression is not apparent in the endoderm or yolk sac (Farin, Imakawa et al. 1990).

During the early stages of pregnancy, it is well established that progesterone stimulates the production of the endometrial secretions necessary for embryo development. The effects of this progesterone include increased endometrial protein secretion (Garrett, Geisert et al. 1988) and increased production of PGE_2 (Vincent, Meredith et al. 1986). In cows, maternal concentrations of progesterone have a marked influence on the development of the embryo (Mann, Mann et al. 1996) and its ability to produce interferon-τ (Mann, Lamming et al. 1998). Cows with a late post-ovulatory increase in progesterone or lower luteal phase concentrations had embryos that, on day 16, exhibited little or no elongation and produce little or no interferon-τ. Conversely day 16 embryos of cows with an earlier rise in progesterone to higher luteal phase concentrations were well elongated (> 4 cm) and produced large quantities of interferon-τ. These findings suggest that an early increase in progesterone is

more important in stimulating embryo development and interferon-τ synthesis than are later progesterone concentrations (Mann, Lamming et al. 1999).

2.1.3 The Maternal Signal—Development of the luteolytic Mechanism

In cattle, concentrations of endometrial oxytocin receptors are low or undetectable from about day 6-8 of the luteal phase to immediately before luteolysis, about day 15-17, when concentrations begin to increase (Meyer, Mittermeier et al. 1988; Fuchs, Behrens et al. 1990; Mann and Lamming 1994). The pulsatile secretion of luteolytic $PGF_{2\alpha}$ begins on about day 17 and is associated with a small rise in endometrial oxytocin receptor concentration (Mann and Lamming 1993). By collection of repeated biopsy samples of uterine endometrium, Mann *et al* (1999) found that oxytocin receptors, which are undetectable through much of the luteal phase (< 20 fmol/mg protein), rise to a concentration of 121 ± 16 fmol /mg protein when large luteolytic episodes of $PGF_{2\alpha}$ secretion are first observed. The onset of $PGF_{2\alpha}$ secretion is followed by luteolysis within 48 h and oxytocin receptor concentrations continue to increase to maximum concentrations of 500-1000 fmol /mg protein at estrus. Furthermore, a marked increase in oxytocin-induced $PGF_{2\alpha}$ production occurs in heifers between day 13 and day 16, despite only a slight increase in concentration of endometrial oxytocin receptors; a large increase in oxytocin receptor concentration did not occur until day 19. Thus luteolytic $PGF_{2\alpha}$ release requires only a modest increase in uterine oxytocin receptors. The peak concentration of oxytocin receptor obtained at estrus are associated with the fall

in progesterone and increase in oestradiol secretion that occur as a result of luteolysis and are not, therefore, the cause of luteolysis (Mirando, Becker et al. 1993).

In cows oxytocin receptors first appear on the luminal epithelium of the uterine endometrium (Robinson, Mann et al. 1999). This occurs at the same time as the uterus develops the ability to release $PGF_{2\alpha}$ in response to oxytocin (Mann and Lamming, 1994). This finding demonstrates that, in cows, it is the development of oxytocin receptors on the luminal epithelium that is the key event in the development of the luteolytic mechanism. The initiation of the luteolytic mechanism requires only a relatively small increase in endometrial oxytocin receptors and it is this initial increase in oxytocin receptor development within the luminal epithelium which the embryo must counteract if it is to prevent luteolysis.

2.1.4. Uterine oxytoxin receptors during early pregnancy

The initial small rise in oxytocin receptors, localized to the luminal epithelium of the endometrium, is the event in the initiation of luteolysis. Thus the inhibition of this initial rise in oxytocin receptors appears to be the key event in the establishment of pregnancy. Fuchs *et al* (1990) found that the presence of a viable conceptus in the uterus completely prevented both the small rise in oxytocin receptors in the uterus between day 14 and day 17, and the much larger rise seen between day 17 and day 21 in cyclic cows. On day 16, a time at which the luteolytic mechanism is beginning to develop, oxytocin receptor mRNA was

detectable in the luminal epithelium of over 40% of non-pregnant cows but was undetectable in all cows with an embryo (Robinson *et al.*, 1999).

2.1.5. Maternal Recognition of Pregnancy

Antiluteolytic effects of IFN-τ are responsible for maternal recognition of pregnancy, which is the term used to describe how a mother responds (physiologically) to the presence of a conceptus in her reproductive tract. In domestic ruminants, the developing embryo does not implant until relatively late in development, although the conceptus is clearly capable of communicating with the mother well before implantation occurs, and before the conceptus has access to the maternal circulation. Failure of the conceptus to signal its presence at the appropriate time leads to pregnancy loss (Demmers, Derecka et al. 2001).

The principal role of the embryo during the maternal recognition of pregnancy is to inhibit the development of oxytocin receptors on the endometrium and hence the release of $PGF_{2\alpha}$ that is responsible for the demise of the corpus luteum. In sheep, it has been postulated that interferon τ prevents the rise in endometrial estrogen receptors that is thought to precede the rise in oxytocin receptors necessary for the induction of luteolytic release of $PGF_{2\alpha}$. This contention is supported by several studies (Spencer and Bazer 1995). However, studies in cows indicate that the initial inhibition of oxytocin receptor development on the luminal epithelium and luteolytic $PGF_{2\alpha}$ release occur in the absence of an effect on estrogen receptor concentrations (Robinson et al., 1999).

It is important to consider the control of $PGF_{2\alpha}$ release. One such mechanism in cows involves the stimulation, by the embryo, of an endometrial inhibitor of $PGF_{2\alpha}$ synthesis (Thatcher, Meyer et al. 1995). In sheep, pregnancy is associated with a reduction in pulsatile release of $PGF_{2\alpha}$ coupled with an increased basal secretion, i.e. a change in the pattern and not the quantity of $PGF_{2\alpha}$ secretion. However, in cattle the attenuation of luteolytic episodes occurs in the absence of an increase in basal secretion of $PGF_{2\alpha}$ (Thatcher *et al.*, 1995). This species difference is clearly supportive of the presence of a direct inhibitory effect of the embryo on $PGF_{2\alpha}$ secretion in cows.

During the early stage of pregnancy, progesterone induces differentiation and maturation by acting on the estrogen-primed endometrium through progesterone receptors. This results in switching on of several progesterone dependent genes. Genes, negatively regulated in the estrogen dominant phase of estrous cycle, may also be expressed. This results in synthesis of a number of endometrial proteins and other factors and consequently alters the structural and molecular profiles of endometrium (Hegele-Hartung, Mootz et al. 1992; Beier-Hellwig, Bonn et al. 1994). Thus, structural and biochemical remodelling of endometrium will be discussed in the following sections.

2.2. Structural and biochemical remodelling of endometrium in the estrous cycle and early gestation

Endometrium has been a focus of extensive research investigations because of its crucial functional relevance in various female reproductive events

i.e. estrous cycle, implantation and maintenance of pregnancy. After pregnancy recognition, maintenance of pregnancy requires reciprocal communication between the conceptus and endometrium during implantation and synepitheliochorial placentation (Wimsatt 1950). The endometrium undergoes several structural and biochemical changes in every estrous cycle and pregnancy to facilitate embryo implantation.

2.2.1. Structural remodelling

The uterus of the domestic mammal consists of a corpus (body), a cervix (neck), and two horns. The mucous membrane lining the uterus is a highly glandular structrure, the *endometrium*. It varies in thickness and vascularity with hormonal changes in the ovary and with pregnancy (Frandson and Spurgeon 1992). Cattle have a 21 day estrous cycle governed by follicular estrogen and luteal progesterone. These hormonal shifts allow for the development and ovulation of an egg, receptivity to mating and preparation of the uterus for pregnancy. In the uterus, intercellular communication requires integration of hormonal signals from the ovaries and fetus, which in turn depends on tissue and cell specific expression of steroid receptors (Kimmins and MacLaren 2001). The mechanisms which govern such interactions in the uterus include cell-cell contact, paracrine transmission of hormones, cytokines and signalling molecules, and extracellular matrix and cell adhesion molecule interactiona at tissue boundaries (Bartol, Wiley et al. 1999; Roberts, Ealy et al. 1999). The tissue

distribution of steroid receptors is regulated during the estrous cycle and early pregnancy in a cell specific manner (Kimmins and MacLaren 2001).

The ruminant placenta shows a considerable uniformity of gross structure across the genus, a flat apposition of trophoblast (chorionic epithelium) to uterine epithelium with a variable number of placetomes. Implantation is a dynamic process requiring intricate signalling interactions between maternal endometrium and fetal trophoblast cells, and remodelling of the endometrium to accommodate placental development. Cattle and other rumimants undergo a relatively non-invasive placentation process that gives rise to a synepitheliochorial placenta. About Day 19-20 of pregnancy, trophoblast attachment begins in the region of the embryonic disk, and binucleate cell migration begins (King 1980; Wathes 1980). These cells arise from the trophoblast, migrate across the microvillar junction, and fuse with maternal epithelial cells to form a hybrid epithelium (Wooding 1992). Changes in the endometrial stroma have been reported during ruminant implantation, including structural changes and angiogenesis as a prerequisite to cotyledon formation (King 1980; Renolds 1992). In sheep, superficial implantation and placentation is a lengthy process that begins on Days 15-16 and is not completed until Days 50-60 of pregnancy (Guillomot 1995). During this period, the ovine uterus grows substantially in order to accommodate rapid conceptus development and growth in the latter half of pregnancy. In addition to placental development in the caruncular areas of the endometrium and changes in vascularity, the intercaruncular endometrial glands grow substantially

in length (four-ford) and width (ten-ford) during pregnancy in ewes (Stewart 2000).

Histomorphological analysis of non-receptive endometrium from antiprogestin treated animals clearly indicated that blocking the progesterone action induces retardation in endometrial development (Rosario, Sachdeva et al. 2003). Thus, the expression of the molecular markers could play a significant role in endometrial growth, development and thereby receptivity.

2.2.2. Biochemical remodelling

2.2.2.1 Steroid hormone receptors

It has been now unequivocally established that ovarian steroids—progesterone (P4) and estrogen (E2) are the major determinants of morphological and functional maturation of endometrium. Both E2 and P4 are known to paly critical roles in regulating the endometrial growth and development in cyclic manner. While E2 induces growth and proliferation of endometrium, P4 induces endometrium to undergo differentiation and maturation (Rosario, Sachdeva et al. 2003). Ovarian hormones mediate their activity via specific receptors on endometrium. E2 is known in inducing the synthesis of both estrogen (ER) and progesterone receptors (PR) while P4 down-regulates the expression of ER and PR (Fujishita 1997). P4 once bound to PR, initiates a series of events, which leads to synthesis of various cytokines, growth factors etc (Rosario, Sachdeva et al. 2003).

A number of studies in both cattle and sheep have demonstrated the general ability of the embryo itself, embryo-derived interferon-τ or recombinant interferon-τ to inhibit the development of oxytocin receptors on the endometrium. It is now generally accepted that this is the event in the maintenance of pregnancy in both species.

As discussed previously, induction of OTR is dependant on the action of E2 and P4. The effects of pregnancy on endometrial oxytocin, estradiol and pregesterone receptors on day 16 in non-pregnant cows and in cows with an embryo present in the uterus have been investigated (Robinson *et al.*, 1999). In pregnant cows, there was a significant inhibition of both endometrial oxytocin receptor mRNA concentrations and oxytocin-induced secretion of $PGF_{2\alpha}$. However, despite the inhibitory effect of the embryo on the initiation of a luteolytic mechanism, measurement of both estradiol receptor mRNA and estradiol receptor protein revealed no differences between pregnant and non-pregnant cows. Thus it would appear that in cows, the embryo can inhibit both the initiation of oxytocin receptor and oxytocin-induced secretion of $PGF_{2\alpha}$ without affecting estradiol receptor concentrations. As with the estrogen receptor, progesterone receptors were also present at similar concentrations in both pregnant and non-pregnant cows in all regions of the uterus studied, supporting the idea that changes in endometrial progesterone receptor concentrations are not involved in the inhibition of luteolysis during pregnancy (Robinson, Mann et al. 2001).

2.2.2.2. IFN-τ

IFN-τ induces the expression of a number of genes, such as STAT (signal transducer and activator of transcription) 1 and 2 (Stewart, Johnson et al. 2001), IFN-regulatory factor 1 (IRF-1) (Spencer, Ott et al. 1998), ubiquitin crossreactive protein (Johnson, Austin et al. 1998; Johnson, Spencer et al. 1999), Mx protein (Ott, Yin et al. 1998), granulocyte-chemotactic protein-2 (Teixeira, Austin et al. 1997), 2', 5'-oligoadenylate synthetase (Johnson, Stewart et al. 2001). Further, IFN-τ stimulates the expression of granulocyte-macrophage colony-stimulating factor, a cytokine with putative positive effects on the conceptus, in stromal cells of the endometrium (Emond, Asselin et al. 2000). Other effects of IFN-τ in endometrium cells include a reduction of oxytocin-induced cyclooxygenase-2 and prostaglandin F synthetase expresstion (Xiao, Murphy et al. 1999). The regulation of those proteins by IFN-τ may have important implications for cytokine networking in the uterus during pregnancy. Also, the regulation of inflammation and angiogenesis by those proteins with other cytokines may be integral to establishing early pregnancy and implantation in the cow (Teixeira, Austin et al. 1997) and to the maintenance of early pregnancy in ruminants (Johnson, Austin et al. 1998).

2.3. Early Gestation

Gestation is achieved through an array of events that include fertilization, attachment, implantation and placentation. Implantation and consequent placentation are important processes for success of gestation as remarkable

changes occur between the conceptus and uterine endometrium (Wooding 1992; Weilauf 1994). In the cow, the elongation of the conceptus starts around two weeks after fertilization and it first attaches to the endometrium at about three weeks of gestation (Wooding 1992).

2.3.1 Implantation

Implantation refers a series of highly coordinated interactions that begin with intimate contact between apical plasma membranes of the conceptus trophectoderm and the uterine luminal epithelium and conclude with the formation of placenta as a means to support embryonic/fetal development throughout pregnancy. Before any intercellular contacts are established, secretions from the embryo and uterine endometrium exert a mutual influence to support further development of the conceptus. Embryonic secretory signals sustain the function of the corpus luteum during the early stages of pregnancy (Hafez 1993)

Implantation begins with an uncommon union between apical plasma membranes of the two genetically distinct tissues (i.e. the embryo and the uterine epithelium). The process of implantation in domestic animals (i.e. cattle, sheep) differs in a number of important ways from implantation in rodents and primate species. In contrast to rodents and primates, where the embryo attaches almost immediately to the uterine epithelium upon entering the uterus, domestic animals have a prolonged pre-implantation period upon arrival of the embryo in the uterus. Rather, the pre-implantation period is characterized by endometrial gland

secretion, and the generation of the conceptus signal for maternal recognition of pregnancy. Further, the trophoblast with its supporting layer of extra-embryonic mesoderm is simply apposed to the uterine epithelium in domestic animals: i.e. there is little invasion of the maternal tissue by fetal tissue and the conceptus remains within the uterine lumen throughout gestation (epitheliochorial implantation) (Hafez 1993).

In the cow, there is evidence that the expression of carbohydrate determinants and cell surface antigens change in the pre-implantation conceptuses (Dowsing, Gougoulidis et al. 1998) and it is anticipated that protein and glycoprotein expression at the surface of the uterine epithelium is also modulated at this time (Skinner, MacLaren et al. 1999).

2.3.2. Placentation

The endometrium provides a mechanism for attachment of the extraembryonic membranes. This union forms the placenta, and the process is called placentation. As illustrated in Figure 1, the bovine placenta is multiplex, villous and epitheliochorial. It forms 80 – 120 placentomes [Figure 1(B)], areas in which tufts of chorionic villi (cotyledons) attach to crypts developed from preformed endometrial prominences (caruncles). With formation of the placenta, nutrients from maternal blood can be transferred to embryonic and fetal blood and waste products from embryonic and fetal blood can be eliminated through the maternal systems (Bearden and Fuquay 2000). Cows have *cotyledonary* placental attachments. *Chorionic villi* from the extraembryonic membranes

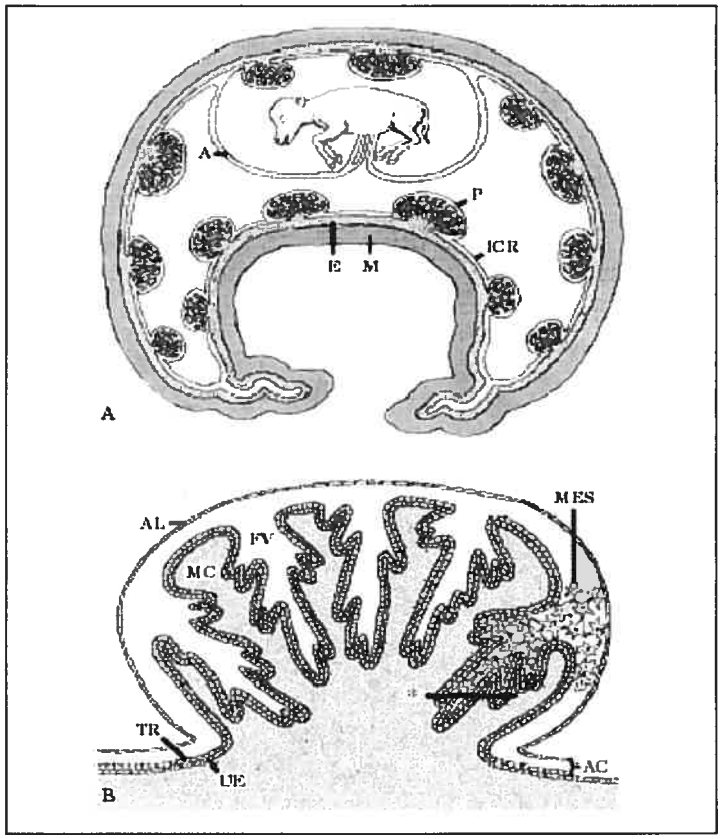

Figure 1. (A) Schematic drawing of bovine fetus in utero. The grey part represents the maternal compartment composed of the endometrium (E) and the myometrium (M). Allantoamniotic membrane (A); placentome (P); intercotyledonary region (ICR). (B) Detailed schematic drawing of a placentome. The allantochorion (AC) is smooth in the ICR, which apposes likewise smooth E. In the placentome, the AC is called cotyledon and forms fetal villous (FV) trees that fit in maternal crypts (MC). In the AC, a loose network of mesenchymal cells (MES) is covered with allantoic endoderm (AL) toward the allantoic cavity and with an intact trophoblast cell (TR) layer opposing the uterine epithelium (UE). Adapted from (Schauser, Nielsen et al. 2001)

penetrate into carunles, which are button-like projections on the endometrium. This union, chorionic villi and carunles, forms the placentome (also called the cotyledon) (Bearden and Fuquay 2000).

Binucleate cells are found in the fetal trophectodermal epithelium of all ruminant placentas. They are present in fairly constant proportions from implantation to parturition. The placental lactogen system in the sheep placenta is restricted to the binucleate cells. Counts of the frequency of binucleate cells and their migration indicated that most, if not all, binucleate cells migrated to the lumenal epithelia in glandular areas of the uterus (caruncles). The result of the migration is fusion of a binucleate cell with a uterine epithelial cell or a syncytial layer. This fusion delivers the characteristic binucleate cell granules close to the maternal circulation while maintaining the trophectodermal barrier to other feto-maternal exchange. The ruminant binucleate cell therefore seems to play a central role in forming the structures and secretions at the feto-maternal interface which may be crucial in establishing and maintaining pregnancy (Wooding 1982).

2.3.3. Embryo Development

After fertilization, the zygote will divide many times without any increase in cytoplasm. The overall size may increase due to absorption of water, but the total cellular material will decrease. This process of cell division without growth is cleavage. The first cleavage will result in a 2-cell embryo. This is followed by additional cleavages resulting in 4-cell, 8 cell, 16-cell, 32-cell embryos, and

so on. With each cleavage, cells become smaller. In cows and ewes, an 8 to 16-cell embryo will enter the uterus 3 to 4 days after ovulation. The 8 to 16-cell stage embryo is called a morula. By the 32 to 64 cell stage, the morula will compact with gap junctions forming between interior cells and tight junctions forming between cells on the outside of the embryo, a necessary step in blastocyst formation. The blastocyst stage will be reached by day 7 in cattle. The development that occurs in the oviduct is critical to survival of the embryo (Killian 2004).

2.3.4. Differentiation

It is a period when the cells are in the process of forming specific organs in the body of the embryo. Notable events during differentiation include the formation of the germ layers, extraembryonic membranes, and organs. In addition, rapid changes in relative size occur during differentiation. This will occur by day 12 in cows (Peters 1987). After differentiation is completed, the product of conception is called a fetus rather than an embryo. This portion of gestation, between the completion of differentiation and parturition, has been termed the "the period of the fetus." The principal development feature of this period is growth (Hafez 1993; Bearden and Fuquay 2000).

As reviewed previously, the uterus undergoes biochemical remodelling during pregnancy. Macrophage migration inhibitory factor (MIF) expression and secretion are regulated by endocrinological changes during pregnancy in some species and maybe important for establishment of pregnancy in ruminants

(Suzuki, Kanagawa et al. 1996). The features and functions of MIF in reproduction will be discussed in next section.

2.4 Macrophage migration inhibitory factor (MIF)

2.4.1 Cytokines

Cytokines are a group of regulatory proteins with a MW of less than 100 kDa secreted by cells of the immune system and act nonenzymatically in picomolar to nanomolar concentrations (Abbas, Lichtman et al. 1994). Cytokines play a pivotal role in regulating the host inflammatory and immune responses to infection and tissue invasion (Kauma 2000). They regulate the first nonspecific phase of the host response by orchestrating a local inflammatory reaction and then serve to control the subsequent specific immune response. Structurally cytokines are small α–, α/β–, or β–proteins with a molecular weight of 8-30 kDa that can often be grouped into subfamilies according to their structure or the structure of their corresponding receptors. Examples include the interleukin (IL) 6 family of cytokines and receptors, the chemokine family, and the tumor necrosis factor (TNF)-Fas ligand-CD30 ligand-CD28 ligand family. Cytokine biological activities are both pleiotropic and redundant, indicating that the molecular interplay leading to the balanced functioning of immune system is very complex. Novel members of the various cytokine families continue to be discovered, further adding to the complexity of the cytokine network (Durum and Oppenheim 1993; Thompson 1993).

Cytokine production and action are largely restricted to cells of the immune system. However, it also has become clear recently that specific cytokine production and cytokine effects can occur in other cell types. An immune-neuroendocrine network of cytokine action has been shown to participate in the regulation of inflammatory reactions and in the general host stress response. Research programs aimed at elucidating the pathways of immune-neuroendocrine regulation on a molecular level have demonstrated that systemic release of endocrine hormones, particularly glucocorticoids, may act to modulate immune system reactivity, and in turn that mediators of the immune response, i.e., cytokines, may serve to regulate neuroendocrine functions (Goldstein, Bowen et al. 1992; Spangelo and Gorospe 1995).

The maintenance of a state of physiological equilibrium within the host requires the interplay of various processes that have both complementary and opposing functions. Bernhagen et al (1998) initiated an exploratory research program to identify novel mediators that might be released systemically, and that can modulate inflammatory and immune responses. These studies have led to the identification of migration inhibitory factor (MIF), a previously known T cell cytokine of largely unknown function, as a critical component of the immune system and counter-regulator of glucocorticoid action with unusual structural and functional features (Bernhagen, Calandra et al. 1998).

2.4.2 Discovery, cloning and structure of MIF

MIF was identified nearly four decades ago as one of the first cytokines discovered (Bloom and Bennett 1966). MIF was initially described as an immune activity isolated from the supernatants of T lymphocytes and was found to inhibit the random migration of macrophages and subsequently to activate macrophage function. Over the years, MIF activity was associated with macrophage phagocytosis and delayed-type hypersensitivity (Nathan, Karnovsky et al. 1971). Research on MIF has been hampered because the entity responsible for the observed immune activities was not defined at a molecular level for almost three decades.

Today, MIF is cloned (Bernhagen, Calandra et al. 1993) and its structure has been well characterized by crystallization, nuclear magnetic resonance spectroscopy and various biochemical methods. The structural properties of MIF have recently been reviewed (Bernhagen, Calandra et al. 1998) and shall thus not be discussed in detail here.

2.4.3 Inhibition of macrophage migration by MIF

In 1966 historical experiments by Bloom and Bennett (Bloom and Bennett 1966) and David (David 1966) first identified MIF as a nondialyzable protein factor produced by sensitized lymphocytes and which was associated with delayed-type hypersensitivity (DTH). MIF was characterized by the activity of crude extracts to inhibit the random migration of guinea pig peritoneal exudates macrophages in vitro (Bloom and Bennett 1966; David 1966) and

subsequently to activate macrophage function (Nathan, Karnovsky et al. 1971; Nathan, Remold et al. 1973). In spite of these observations that described a cytokine activity more than 30 years ago, a detailed view of the biological role of MIF has remained elusive until very recently. This was due in large part to the failure to identify the molecular entity that is associated with the observed inhibition of macrophage migration. It is now clear that other immune factors such as interferon-γ and IL-4 also exhibit migration inhibitory effects, and that these cytokines were also present in the complex cellular supernatants that had been used in the initial MIF studies (Thurman, Braude et al. 1985; McInnes and Rennick 1988).

The cloning in 1989 of a human T cell protein with a molecular weight of 12.5 kDa unraveled the identity of MIF as this molecule was distinct from previously discovered cytokines with MIF activity (Weiser, Temple et al. 1989). However, the lack of biologically active, purified recombinant protein and the identification of a mitogenic contaminant within the original recombinant MIF preparations slowed further research in the field. As a result it was unclear at that time what the precise relationship is between the 12.5-kDa MIF protein and the various migration inhibitory assays that have been applied over the years.

2.4.4 Main characteristics and biological activities of MIF

MIF exhibits a number of unusual properties that distinguish this factor from other cytokines. The most important of these features are summarized here. MIF is considered a pleiotropic lymphocyte and macrophage cytokine, but

numerous reports (Waeber, Calandra et al. 1999; Fingerle-Rowson and Bucala 2001) also suggest that MIF is an endocrine factor. Intriguingly, MIF has been demonstrated to have at least two distinct catalytic activities, i.e. a tautomerase and an oxidoreductase activity. Accordingly, MIF has been termed "cytokine with enzymatic properties or cytozyme" and "secreted enzyme". As one of the catalytic activities found is reminiscent of the oxidoreductase activities of the thioredoxin family of proteins, MIF has recently been coined "redoxkine" (Ghezzi P. et al., 2000). The physiological relevance of the reported enzymatic activities of MIF is not yet resolved.

MIF is ubiquitously expressed in both immune and non-immune cells including various peripheral tissues. Its most critical functions encompass the regulation of macrophage function (Calandra, Bernhagen et al. 1994; Onodera, Suzuki et al. 1997), lymphocyte immunity (Bacher, Metz et al. 1996; Abe, Peng et al. 2001) and endocrine functions (Calandra, Bernhagen et al. 1995; Meinhardt, Bacher et al. 1996; Waeber, Calandra et al. 1997; Bacher, Meinhardt et al. 1998). MIF is a unique counter-regulator of the immunosuppressive and anti-inflammatory activities of glucocorticoids (Calandra, Bernhagen et al. 1995; Daun and Cannon 2000).

One other non-typical property is that MIF is effectively secreted from a variety of immune and some non-immune cells without having an N-terminal leader sequence or an apparent internal signal sequence for import into the endoplasmic reticulum. It has thus been concluded that MIF is secreted by a

non-conventional leaderless pathway (Bernhagen, Calandra et al. 1998). However, the precise mechanism of secretion has not yet been elucidated.

2.4.5 Role of MIF in cell proliferation, angiogenesis and tumorigenesis

A number of recent studies imply that MIF could be centrally involved in processes regulating cell proliferation and tumor angiogenesis (Takahashi, Nishihira et al. 1998); (Yang, Degranpre et al. 2000). Moreover, Hudson et al. suggest that cell cycle regulation by MIF could be related to MIF's inflammatory activity (Hudson, Shoaibi et al. 1999).

In an attempt to explain the increased expression of cytosolic MIF in murine colon carcinoma cells in response to growth factors, Takahashi et al. (1998) investigated the correlation between the expression of MIF and cell proliferation and found that MIF expression was associated with enhanced proliferation of these cells. Chesney et al. demonstrated that neutralizing anti-MIF-antibodies dramatically reduced the initial outgrowth of 38C13 B cell lymphoma cells in C3H/HeN mice (Chesney, Metz et al. 1999). As immune neutralization of MIF did not significantly affect the growth of established tumors, Lue and Kleemann suggested an early primary effect for MIF. They subsequently showed that neutralization of MIF by anti-MIF antibodies inhibited endothelial cell growth and led to a reduced number of tumor capillaries, but did not affect the proliferation of the lymphoma cells (Lue, Kleemann et al. 2002). In line with these observations, inhibition of tumor angiogenesis by anti-MIF antibody treatment was reported in a human melanoma model (Shimizu, Abe et

al. 1999) and in murine colon carcinoma cells (Takahashi, Nishihira et al. 1998) and (Ogawa, Nishihira et al. 2000). In addition, Yang and coworkers identified MIF as an angiogenic factor released by ectopic human endometrial cells promoting human coronary artery endothelial cell growth (Yang, Degranpre et al. 2000). Recombinant MIF enhanced the effect of a cocktail of growth-stimulating factors but not alone, and MIF antibody reduced the stimulatory effect of the growth factors, suggesting together that MIF has an indirect effect on cell proliferation induced by growth factors, but may not act in a proliferative manner itself. The studies also imply that increased cytosolic MIF expression in tumors is linked to the proliferative properties of tumor cells, a notion that is confirmed by the finding that over expression of antisense MIF constructs led to an inhibition of cell proliferation (Takahashi, Nishihira et al. 1998). However, del Vecchio and colleagues studied MIF levels in different stages of prostatic adenocarcinomas (del Vecchio, Tripodi et al. 2000) and found that MIF expression was stronger in low-grade than in high-grade adenocarcinomas, indicating that with histological dedifferentiation, prostate adenocarcinoma cells show a reduced MIF expression and that MIF expression, while generally elevated in tumors could be inversely related to tumor development. In fact, the observation by del Vecchio and colleagues is consistent with the proposed early primary effect of MIF on tumor cell proliferation indicated by the study of Chesney et al. (1999).

Together, the recent studies suggest that modulation of cell proliferation by MIF could involve a complex regulatory system in which the proteins

JAB1/CSN5 and p53 and possibly other signalosome proteins may be involved (Bacher, Meinhardt et al. 1998; Hudson, Shoaibi et al. 1999; Kleemann, Hausser et al. 2000; Bech-Otschir, Kraft et al. 2001). Although this evidence is circumstantial at this point, detailed future studies will have to focus on these proteins to clarify their importance in MIF-mediated regulation of apoptosis, cell proliferation and tumorigenesis.

2.4.6 Role of MIF as a T cell cytokine

For almost 30 years MIF was considered to be a T cell cytokine released upon lymphocyte-specific stimulus. The macrophage was considered to be its main cellular target. Accordingly, the naming of MIF referred to this activity (Bloom and Bennett 1966; David 1966). Despite the recent discovery of pituitary and macrophage MIF serving crucial functions as proinflammatory mediators of sepsis and other inflammatory diseases, work performed over the past 10 years also has demonstrated that T cell MIF plays an important role in the regulation of immunity.

MIF is an abundant preformed constituent of resting primary mouse T cells and human and mouse T cell lines. Resting T cells also contained measurable amounts of MIF mRNA. By enzyme-linked immunosorbent assay the MIF protein content in these cells was determined to be 4 fg/cell and 170 fg/cell in primary T cells and T cell lines, respectively (Nishino, Bernhagen et al. 1995). This is similar to the values observed in primary monocytes (2 fg/cell) and macrophage cell lines (120 fg/cell) (Bacher, Metz et al. 1996).

T cell activation by specific antigen, mitogens, or anti-CD3 antibodies has been found to lead to increased MIF mRNA expression and secretion of MIF protein, indicating that MIF production is also an important feature of T cell activation (Bacher, Metz et al. 1996). From a historical perspective, T cells serve as a source of MIF, the macrophage being its main target. Nevertheless, very little had been described concerning the effect of purified recombinant MIF on lymphocytes. It has now shown that MIF is a critical component of both the T cell and B cell activation pathways by inhibiting T cell proliferation and B cell antibody production in vivo (Bacher, Metz et al. 1996).

An interesting aspect of T cell MIF biology also has came from work on so-called T suppressor factors. A molecule termed glycosylation inhibition factor that had been found to be expressed in certain T suppressor cells and to participate in the inhibition of the glycosylation of an IgE binding protein is identical in primary structure with MIF (Liu, Nakano et al. 1994).

DTH reactions are mediated by pathways central to cellular immunity. T cells and monocytes/macrophages are the predominant infiltrating cell populations found in DTH sites. Cytokines play a pivotal role by regulating cellular infiltration into DTH sites and immune cell activation. MIF was the first cytokine demonstrated to be associated with DTH (Bloom and Bennett 1966; David 1966). As mentioned above, MIF was thought to be released by T cells that are localized at DTH sites and to act to contain infiltrating monocytes/macrophages at the inflammatory locus. Some studies on the role of MIF in the classic tuberculin-induced DTH response in the mouse indicate that

the macrophage rather than the T cell is the predominant source of MIF protein and mRNA expression within DTH inflammatory sites (Bernhagen, Bacher et al. 1996). The data showing that MIF is an important component in the pathway leading to T cell activation suggest that, once released by DTH macrophages, MIF acts to activate the T cells that colocalize at the DTH site. Thus despite the involvement of the macrophage as an important source of MIF these studies confirm overall the important historical observation of MIF as a mediator of the DTH response

2.4.7. MIF as a counter regulator of glucocorticoid action

The information on the biological role of MIF has come from studies on MIF and glucorticoid hormones. As mentioned above, a key feature of the central stress response to infection and inflammation is the induction of endogenous glucocorticoid steroids. Once released, glucocorticoids are potent anti-inflammatory agents and immunosuppressants. As a rule cytokine expression is inhibited by steroids. By contrast, MIF expression by monocytes/macrophages and T cells is induced rather than suppressed by glucocoticoids (Calandra, Bernhagen et al. 1995). MIF release from pituitary cells is also stimulated by glucocorticoid hormone, raising the intruiging possibility that steroid feedback regulation within the HPA axis acts both negatively, by inhibiting further ACTH release (via hypothalamic corticotropic-releasing hormone), and positively, by stimulating MIF secretion. Although not

yet confirmed experimentally, it is likely that glucocorticoid-mediated MIF release by the pituitary is restricted to granule populations that are MIF specific.

MIF secretion by macrophages occurs at low, physiological concentrations of glucocorticoids and decreases at higher (not physiological) concentrations (Figure 2). Furthermore, micromolar concentrations of dexamethasone which are routinely used experimentally to inhibit cytokine expression do not suppress MIF induction that is stimulated by proinflammatory agents such as LPS or TNF-α. The observation that a potent anti-inflammatory agent such as cortisol induces the macrophage and the T cell to secrete a "proinflammatory" cytokine was at first puzzling. This apparent paradox was resolved, however, by a series of experiments which showed that when MIF is added together with dexamethasone, it can overcome, in a dose-dependent fashion, glucocorticoid inhibition of monocyte TNF-α, IL-Iβ, IL-6, and IL-8 secretion. Conversely, when human monocytes are stimulated with LPS in the presence of dexamethasone and neutralizing anti-MIF IgG, TNF-α production is inhibited by as much as 33% (Calandra, Bernhagen et al. 1995). The balance between the glucocorticoid and MIF concentrations was found to be the main determinant of cytokine production in this in vitro system. The ability of MIF to override steroid action was confirmed by in vivo experiments in a mouse model of endotoxic shock. MIF, when administered together with glucocorticoids and 2 h before a lethal dose of LPS, was demonstrated to override glucocorticoid inhibition of LPS lethality (Suzuki, Sugimoto et al. 1996).

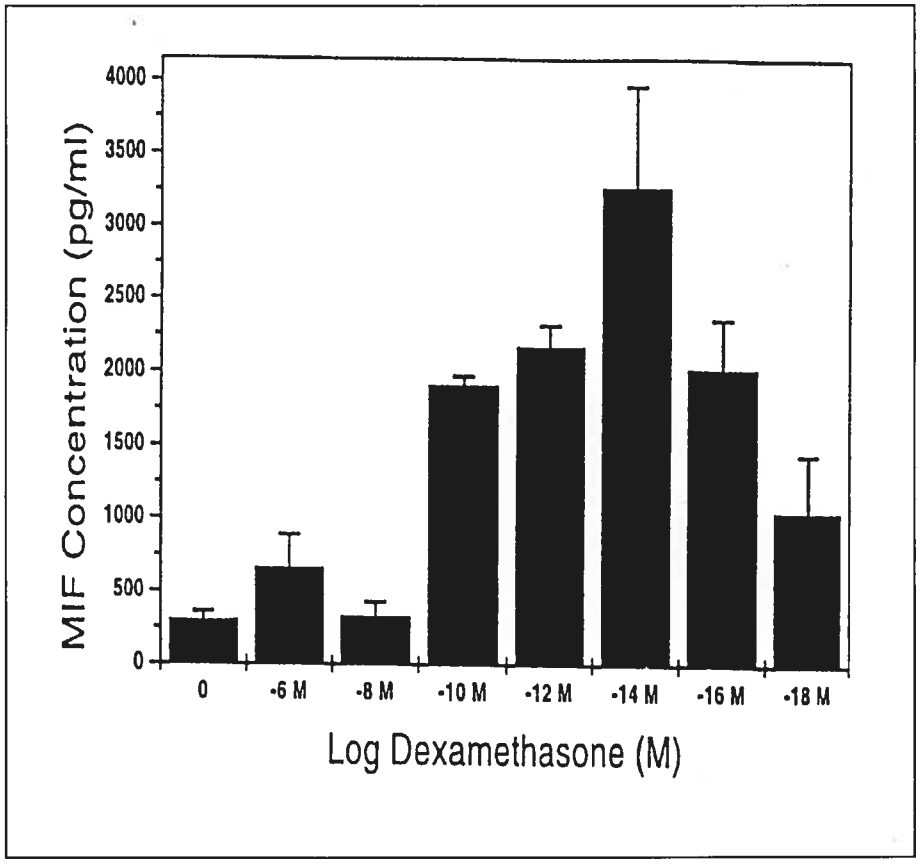

Figure 2. Glucocorticoid-induced MIF secretion by macrophage. Raw 264.7 macrophages were stimulated with dexamethasone at the indicated concentrations and cell-conditioned media analyzed for MIF secretion by enzyme-linked immunosorbent assay. (Adapted from Onodera, Suzuki et al. 1999).

These data suggest therefore that the magnitude of the inflammatory response is a result of the opposing effects of MIF (proinflammatory) and glucocorticoids (anti-inflammatory) on immune cell activation. MIF thus serves as a physiological counterregulatory mediator that counteracts the immunosuppressive effects of glucocorticoids. The localization of MIF both within central tissues (pituitary) and peripherally (macrophages and T cells) and its secretion from these tissues by inflammatory and stress signals thus became readily reconcilable by these findings in the periphery the MIF produced by macrophages and T cells in response to injurious stimuli would antagonize the anti-inflammatory effects of stress-induced in creases in circulating glucocorticoid levels. By counteracting glucocorticoid MIF facilitates the development of the inflammatory response and the mounting of an immune response, should the offending pathogen survive the primary host defense response. The release of pituitary MIF into the circulation indicates furthermore that the host also has the capacity to antagonize the systemic anti-inflammatory properties of glucocorticoid. Of note, the physiological activation of the HPA axis, i.e., by stress handling of experimental rats, leads to an elevation in serum MIF levels that is concurrent with the stress-related increases of the stress hormones ACTH and corticosterone (Calandra, Bernhagen et al. 1995).

2.4.8 Distinct features of MIF

MIF differs from proinflammatory cytokines by a number of characteristic properties. These include both structural and functional aspects, and encompass MIF expression, localization, secretion, and target cell interaction.

With respect to the structure of the MIF gene it is important to note that although MIF shares certain biological features in common with other proinflammatory cytokines such as TNF-α and IL-l, its promoter structure is distinct and lacks several of the *cis* promoter/enhancer motifs, such as the AP-1 and serum-responsive elements that appear to be characteristic of many genes for proinflammatory mediators (Mitchell, Bacher et al. 1995). Furthermore, the MIF gene structure is distinct also in that both cytokine-like and hormone-like motifs such as the nuclear factor-κB binding region and endocrine hormone/negative glucocorticoid responsive element regions, respectively, have been identified (Mitchell, Bacher et al. 1995). The latter is in accordance with the observed role of MIF as pituitary hormone and counterregulator of glucocorticold hormones.

The finding that unstimulated immune cells of the monocyte and T cell lineages contain large, preformed quantities of the cytokine MIF also is noteworthy. Normally cytokine expression in these cells is tightly regulated, and both mRNA and protein expression is induced only upon stimulation. In the case of MIF all stimulation leads to a further increase in cellular mRNA and protein levels accompanied by a concomitant secretion of both the preformed and the newly synthesized proteins, indicating that MIF release is regulated by a

molecular mechanism that is distinct from that of other classically described cytokines.

The proinflammatory activity of MIF has been measured in several test systems. Of these, direct MIF-mediated cell activation is dose-dependent and peaks at 1-5 μg/ml MIF. Such direct biological activities of MIF include MIF-stimulated TNF-α and NO expression by macrophages, the inhibition of macrophage migration, and the activation of macrophage killing toward *Leishmania* parasites. Relatively high concentrations of MIF are observed at baseline in animals (2-5 ng/ml) (Calandra, Bernhagen et al. 1995). Moreover, microgram quantities of MIF have been detected in synovial fluid. Thus, effective proinflammatory MIF concentrations would have to be well above these baseline values and thus might well reach concentrations in the lower microgram per milliliter range in certain pathological situations in vivo.

A tissue survey of MIF mRNA and protein expression has shown that several cell types such as monocytes/macrophages (Calandra, Bernhagen et al. 1994), T cells (Bacher, Metz et al. 1996), anterior pituitary cells (Nishino, Bernhagen et al. 1995), cells of the developing eye lens (Wistow, Shaughnessy et al. 1993), fibroblasts (Lanahan, Williams et al. 1992), and certain parenchymal cells within the skin, liver, brain, pancreas, kidneys, reproductive organs, and adrenals (Meinhardt, Bacher et al. 1996; Suzuki, Kanagawa et al. 1996; Bacher, Meinhardt et al. 1998) constitutively express MIF. Nevertheless, MIF expression is not ubiquitous. For example, granulocytes which, as with macrophages, are a prominent part of the initial host inflammatory response to

invasion, are MIF negative (Calandra, Bernhagen et al. 1994). Furthermore, MIF expression in nonimmune tissues appears to be distinctly restricted to selected cell types. Despite its widespread expression MIF release is tightly regulated, as demonstrated by its stimulus- and cell-specific secretion from monocytes/macrophages, pituitary cells, and T cells (Bernhagen, Calandra et al. 1993; Calandra, Bernhagen et al. 1994; Bacher, Metz et al. 1996).

An important clue about the biological function of MIF is derived from an investigation demonstrating that MIF counteracts glucocorticoid action. Unlike other cytokines, MIF release is induced rather than inhibited by glucocorticoid. Conversely, MIF has been shown to override steroid suppression of the host inflammatory and immune responses in vitro and in vivo (Calandra, Bernhagen et al. 1995; Bacher, Metz et al. 1996). Normally glucocorticoids inhibit cytokine expression resulting both in a local and systemic suppression of cytokines. MIF circulates normally in a concentration (6-25 ng/ml) that is within the range of the glucocorticoid cortisol (approximately 3 ng/ml), indicating that the baseline state of the MIF/glucocorticoid is one of an "active" balance between pro- and anti-inflammatory effects. As for glucocorticoid, serum concentrations of MIF increase many folds during stress, inflammation, or infection. The observation that MIF release follows a bell-shaped, dose-response curve with respect to microbial toxins, and that its overriding capacity is diminished at high concentrations further suggests the existence of important physiological "control points" within the MIF/glucocorticoid counterregulatory system. No other mediator has yet been found to exhibit similar properties with

respect to its expression and functionality towards glucocorticoid hormones. Thus, MIF appears to be the first cytokine to fulfill the requirements for a physiological antagonist of glucocorticoid action. The molecular mechanism of MIF function has not yet been identified. However, several lines of evidence suggest that MIF is an enzyme and thus may function at least in part by non-receptor-mediated pathways. This is very surprising because despite their pleiotropic activities cytokines are known to use membrane receptors on target cells to initiate intracellular - downstream signaling pathways.

2.4.9 Molecular mechanism of MIF function

With a membrane receptor not yet identified, the principal molecular pathways mediating MIF function remain unknown. The observation that MIF can act as an enzyme opens a new dimension in our understanding of how this protein acts in vivo. Whether MIF mediates some of its biological activities via an enzymatic reaction is of obvious interest. However, at present its true, naturally occurring substrate is unknown. As mentioned above, structural data on MIF have revealed a striking architectural homology to the E. coli enzyme CHMI and to 4-oxalocrotonate tautomerase, a related enzyme from *Pseudomonas pulida* that was also found to catalyze the ketonization of 2-hydroxymuconate (Subramanya, Roper et al. 1996; Suzuki, Sugimoto et al. 1996). Although these observations strongly suggest that MIF catalyzes this or similar isomerization reactions, MIF activity in these enzymatic reactions has not yet been investigated. The CHMI and 4-oxalocrotonate tautomerase active sites

have been reported to be located at the N-terminal end of the molecule, with the N-terminal proline residue exhibiting an unusually low pK value of 6.5 enabling it to act as a proton sink (Subramanya, Roper et al. 1996). Although posttranslationally processed MIF also has a N-terminal proline residue, the lack of any primary sequence identity between MIF and the 2-hydroxymuconate isomerizing enzymes indicates that MIF may react with different substrates. Furthermore, the absence in MIF of arginine residues within the putative N-terminal binding site which have been postulated to interact in CHMI and 4-oxalocrotonate tautomerase with the carboxyl groups of 2-hydroxymuconate, makes it unlikely that MIF catalyzes 2-hydroxymuconate isomerization.

One substrate for MIF was identified in the course of studies (Kleemann, Hausser et al. 2000) into the enzymatic pathways responsible for the late stages of melanin biosynthesis. Two enzymes were described that act to convert the melanogenic substrate 2-carboxy-2, 3-dihydroindoindole-5, 6-quinone (dopachrome) into 5, 6-dihydroxyindole-2-carboxylic acid, and these enzymes are specific for the naturally occurring, L-dopachrome stereoisomer. MIF does not catalyze the conversion of the naturally occurring L-dopachrome. However, the methyl esters of both the D- and L-isomers of dopachrome were found to be good substrates for MIF, indicating that MIF catalyzes the conversion of similar ring-like substrates in vivo. The finding that MIF, a soluble, immunoregulatory mediator, also exhibits tautomerization or isomerization activity is reminiscent of the protein cyclophilin, which is the target for the immunosuppressant drug cyclosporin. Cyclophilin functions intracellularly as a peptidylprolyl *cis-trans*

isomerase but also is secreted in response to proinflammatory stimuli (Schreiber and Crabtree 1992; Sherry, Yarlett et al. 1992). By analogy, the constitutive intracellular expression of MIF in conjunction with its reported enzymatic activity would suggest that the biological activity of MIF is mediated by intracellular enzymatic catalysis. This activity could be independent or in conjunction with the observed, cytokine-like activities of MIF. It will be of great importance to identify the putative MIF membrane receptor and its natural substrates that are subject to MIF-mediated isomerization and subsequently to dissect the molecular mechanisms of the MIF enzymatic and extracellular, cytokine-like activities by detailed structure function analyses.

Further evidence supporting the notion of the "enzyme MIF" has come from structural disulfide analyses of MIF. These studies indicate that MIF acts to participate in redox reactions such as the reduction of insulin disulfides or small molecular weight disulfide substrates in transhydrogenase reactions (Mischke, Gessner et al. 1997). Redox reaction catalysis by MIF is reminiscent of the thioredoxin family of proteins, of which some have been reported to act both as intracellular enzymes and secreted cytokines. A more detailed analysis of these activities must be conducted to define the natural substrates for MIF in these reactions and to investigate the connection between the redox and the observed isomerization activities of MIF.

2.4.10. Role of MIF in reproduction

Male reproduction The systematic analysis of tissue expression of MIF also led to the identification of MIF expression in the reproductive organs of the male rat (Meinhardt, Bacher et al. 1996). In the testis, MIF expression is localized to the androgen-producing Leydig cells. The testicular interstitial fluid also contains significant amounts of MIF. Neither recombinant MIF nor neutralizing anti-MIF antibodies have any in vitro effect on basal or luteinizing hormone (LH)-stimulated androgen biosynthesis. MIF protein and mRNA are also expressed in the epithelial cells of the epididymis with increasing expression towards the epididymal caput. From those cells MIF appears to be released via exocytosis as MIF can be detected in secreted vesicles. Furthermore, MIF was identified as a component of the outer dense fibres (ODF), a cytoskeletal element of the mid and principal piece of the sperm tail ((Eickhoff, Wilhelm et al. 2001). MIF is also likely to arise from the prostate gland, where MIF is expressed in the epithelial cells (Frenette, Tremblay et al. 1998).

Despite these observations, $MIF^{-/-}$ male mice do not display any evident disturbance in their reproductive capacity (Bozza, Satoskar et al. 1999). This suggests that the contribution of MIF to male reproduction is either not crucial or can be compensated for by other molecules.

Female reproduction In the female mouse, MIF mRNA has been found in the ovary, the oviduct and the uterus. The mRNA levels change in the uterus of the pregnant mouse, suggesting that MIF expression is regulated by

endocrinological changes during pregnancy (Suzuki, Kanagawa et al. 1996). MIF is expressed in human endometrium across the menstrual cycle and during pregnancy (Arcuri, Ricci et al. 2001).

As in MIF $^{-/-}$ males, female knockout mice reproduce and are not infertile (Bozza, Satoskar et al. 1999). However, a detailed analysis of their reproductive physiology remains to be performed.

As discussed previously, the endometrium undergoes structural and biochemical remodelling during the estrous cycle and pregnancy. Apoptosis is critical for proper tissue remodelling and the regulation of apoptosis is probably imperative for successful pregnancy. The quick and effective removal of apoptotic cells by tissue macrophages represents an essential process, which prevents the release of self-antigens, and in the case of pregnancy, paternal alloantigens. Recent studies (Abrahams, Kim et al. 2004) have shown that the process of apoptotic cell clearance is not a neutral event, but rather an active one that induces macrophage production of anti-inflammatory cytokines and survival factors. Apoptotic cell clearance is, therefore, necessary for the resolution of inflammatory conditions, which during pregnancy could have lethal consequences. The function of the maternal immune system during implantation and throughout pregnancy is, therefore, an important area of investigation. The section will discuss the role of apoptosis during pregnancy.

2.5 The role of apoptosis in early pregnancy

2.5.1. Apoptosis and implantation

Apoptosis, or programmed cell death, is an intrinsically activated cell suicide pathway that regulates cell turnover in normal tissues and maintains cell elimination during embryonic development. Apoptosis also occurs in neoplastic conditions. This cell process has morphologic and biochemical characteristics distinctive from ordinary necrosis (Kerr, Wyllie et al. 1972; Kontogeorgos and Kovacs 1995; Vidal, Horvath et al. 2001). Regulation of apoptosis is complex and involves a family of related proteins that can promote or inhibit this process. Susceptibility of a cell to undergo apoptosis is controlled through the interaction of bcl-2-related inhibitors (e.g. proto-oncogene product bcl-2) and apoptosis promoters (e.g. bax) (Oltvai, Milliman et al. 1993).

During implantation, apoptosis is important for the appropriate tissue remodeling of the maternal deciduas and invasion of the developing embryo (Uckan, Steele et al. 1997). Although, first trimester trophoblasts are resistant to Fas-stimulation, apoptosis has been described in the trophoblast layer of placentas from uncomplicated pregnancies throughout gestation, suggesting that there is a constant cell turnover at the site of implantation necessary for the appropriate growth and function of the placenta (Ratts, Tao et al. 2000; Levy, Smith et al. 2002). Thus regulation of placental apoptosis is essential for the normal physiology of pregnancy.

However, cell death by apoptosis is not the end of the story, the clearance of apoptotic bodies represents a critical step in tissue homeostasis, preventing the

release of intracellular contents, which may cause tissue damage and the possibility to initiate an inflammatory reaction.

2.5.2. Clearance of apoptotic cells

Different morphological changes accompany the execution of the apoptotic program. Cells first become round and detach from their neighbours. Then, condensation of both the nucleus and cytoplasm occurs without major modification to the other intracellular organelles. Following condensation, nuclear fragmentation and membrane blebbing is observed, resulting in the formation of apoptotic bodies with intact membranes. These morphological changes are a translation of the biochemical modifications, mediated by the activation of the caspase cascade, that are occurring inside of the cell (Abrahams, Kim et al. 2004).

Another important cellular change that occurs during apoptosis is the redistribution of membrane proteins, which will allow macrophages to recognize apoptotic cells and direct the phagocytic process. Several receptors have been implicated in the recognition and engulfment of apoptotic cells (Savill and Fadok 2000; Fadok and Chimini 2001), suggesting that the process of phagocytosis is well regulated and functionally relevant.

As discussed above, implantation and trophoblast invasion is characterized by a progressive, continuous induction of apoptosis in the maternal tissue surrounding the fetus (Piacentini and Autuori 1994). During this period, numerous macrophages are present at the implantation site and this was

originally thought to represent an immune response against the invading trophoblast. Macrophage engulfment of apoptotic cells prevents the release of potentially pro-inflammatory and pro-immunogenic intracellular contents that occurs during secondary necrosis (See Figure 3) (Mor and Abrahams 2003). Due to the allogenic nature of the placenta, this process may be essential for the well being of the fetus. Trophoblast cells are carriers of proteins, which are antigenically foreign to the maternal immune system and if released, as result of cell death, may initiate or accelerate immunological responses with lethal consequences for the fetus. Therefore, the appropriate removal of dying trophoblast cells prior to the release of these intracellular components is critical for the prevention of fetal rejection. Macrophages are a key cellular constituent of this process (Mor and Abrahams 2003).

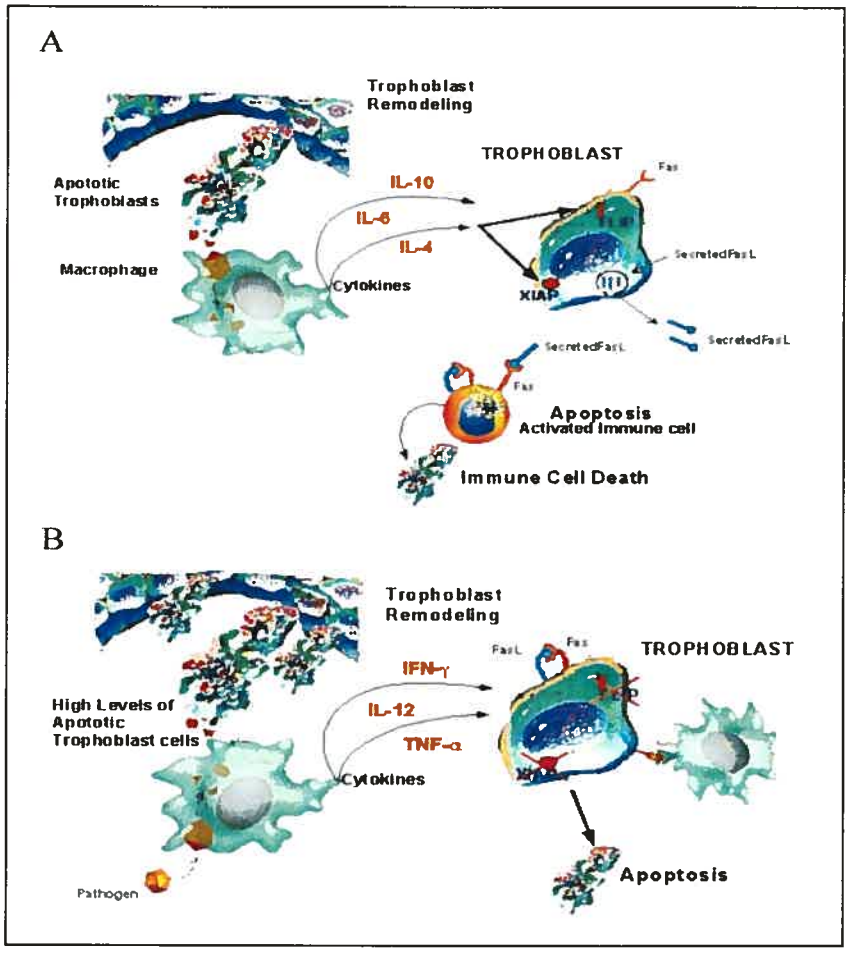

Figure 3. Effect of clearance of apoptotic cells by macrophages. A. Clearance of apoptotic cells induce the expression, by macrophages, of anti-inflammatory cytokines with protective effects on trophoblast survival and immunological tolerance. **B.** Changes in the cytokine milieu, owing to elevated levels of apoptotic bodies and inefficient clearance, will result in a pro-inflammatory microenvironment that in turn may result in changes in trophoblast resistance to Fas-mediated apoptosis and the maternal immune system. Adapted from (Mor and Abrahams 2003).

2.6. Conclusion

The importance of the maternal progesterone profile in the control of the development of the embryo and its ability to produce the anti-luteolytic interferon-τ signal has been clearly established. This is also the case for the role of interferon-τ in the inhibition of endometrial oxytocin receptor up-regulation and subsequent oxytocin-induced luteolytic $PGF_{2\alpha}$ secretion and the inhibition of $PGF_{2\alpha}$ synthesis.

Luteolysis and the prevention of luteolysis are two important events in cyclic and pregnant cows. The luteal regression at the late stage of the luteal phase in cyclic ruminants is caused by an episodic release of $PGF_{2\alpha}$ from the uterine endometrium, which is evoked by oxytocin (OT) after binding to specific endometrial receptors (Bazer et al., 1991). OT receptor number, which is regulated by ovarian estradiol and progesterone, determines the sensitivity of uterine endometrium to OT stimulation. The minor increase in OTR numbers at the late stage of the luteal phase is believed to be responsible for initiating the pulsatile release of $PGF_{2\alpha}$ from the endometrium. Estradiol increases, and progesterone inhibits, ER, PR and OTR. Prolonged (approx. 10 days) exposure of endometrium to high levels of progesterone completely inhibits PR and thus releases the inhibitory effect of PR on ER numbers ('progesterone block'). Without the progesterone inhibition, ER and OTR increase in response to estradiol (McCracken et al., 1984).

In pregnant cows, the establishment of pregnancy requires that conceptus modifies the secretion of PGF from the endometrium, so that regression of CL

does not occur and progesterone secretion is maintained. Overall, what is presently thought to happen during luteolysis and early pregnancy is as follows: estradiol increases the numbers of PR, ER and OTR, while progesterone decreases them. During the first 10 days of the luteal phase, progesterone keeps the ER number low. At the late stage of luteal phase, with the completion of progesterone block, ER number increases and subsequently OTR number increases due to the stimulation by estradiol. The endometrium becomes sensitive to OT and luteolysis is initiated. In the pregnant cow, IFN-τ attenuates $PGF_{2\alpha}$ secretion by inhibiting ER and OTR expression.

The rediscovery of MIF as pituitary hormone and macrophage T cell cytokine that acts to control the host stress and inflammatory response has opened a new dimension to our understanding of the biological function of this mediator. The surprising finding that glucocorticoids induce rather than suppress MIF production, and that MIF functions as a physiological counter-regulator of glucocorticoid action has offered an intruiging biological mechanism of how MIF might participate in the host immune response. It is currently known that MIF is a proinflammatory cytokine that plays an essential role in the activation of T cells. In addition, this protein plays a key role in cell growth with regard to tumorigenesis and embryogenesis. MIF appears to exert its biologic action in the form of either a cytokine, hormone, or enzyme. Munn and Zhou suggested the roles for macrophages of fetal-maternal interface as regulators of maternal T-cell tolerance to the fetal allograft (Munn, Zhou et al. 1998). Hence, it can be

hypothesized that MIF, keeping activated macrophages *in situ*, play an important role in the maintenance of pregnancy.

It is evident that a balance between cell death and proliferation plays a pivotal role in the maintenance of normal tissue homeostasis. This can be particularly important for the successful development of animal pregnancy. Apoptosis within the maternal decidua seems to be important in the establishment of immune privilege in the pregnant uterus to protect foetal cells from killing by maternal cells, whereas in the villous part of the placenta programmed cell death seems to participate in the regulation of placental growth (Hammer and Dohr 1999) .

The apoptosis cascade is regulated in parallel with trophoblast differentiation, syncytial fusion and trophoblast turnover. Differentiation of the placenta depends on continuous incorporation of villous CTB by fusion into STB through apoptotic pathways (Huppertz, Frank et al. 1999). Therefore, apoptosis is a normal physiological process throughout gestation (Smith, Baker et al. 1997). Disturbances in programmed cell death in placenta seems to be associated with abnormal pregnancy outcome. Diminished function of the molecules recruited to the Fas–FasL signaling system may have influence on animal pregnancy outcome (Hunt, Vassmer et al. 1997; Guller and LaChapelle 1999). Taken together, complete understanding of placental apoptotic pathways may be important to understand the pathogenesis of pregnancy loss and other pregnancy related diseases.

3. HYPOTHESIS AND OBJECTIVES

3.1. Hypothesis

IFN-τ modifies protéine secretion from endometrium that are involved in the establishment of pregnancy.

3.2. Objectives

The specific objectives of this project were:

(1) To determine if protein secretion induced by IFN-τ in cultured bovine epithelial and stromal cells is influenced by E2 and P4.

(2) To identifiy and characterize specific proteins secreted from endometrial epithelial cells in response to IFN-τ that could be important for endometrial function and/or embryo development;

(3) To establish an in vitro model to elucidate the effect of IFN-τ on the induction of apoptosis in epithelial cells of bovine endometrium;

4. Article One

Objective

To determine if protein secretion induced by IFN-τ in cultured bovine epithelial and stromal cells is influenced by E2 and P4.

INFLUENCE OF ESTRADIOL AND PROGESTERONE ON PROTEIN SECRETION INDUCED BY RECOMBINANT BOVINE INTERFERON-TAU IN BOVINE ENDOMETRIAL CELLS

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Key words: Bovine, uterus, endometrial cells, steroid hormone

[1] This work was supported by grants from NSERC (AKG)

4.1. ABSTRACT

During early pregnancy in ruminants, interferon-tau (IFN-τ), which is secreted by the embryo, not only prevents prostaglandin F2α release, but it also modifies protein secretion from the endometrium. Numerous reports have identified gene targets influenced by progesterone (P4) and/or estradiol-17β (E2) and have implicated these products as contributors to endometrial physiology or pathology. The objective of this study was to determine if protein secretion induced by recombinant bovine interferon-tau (rbIFN-τ) in cultured bovine uterine epithelial and stromal cells was modified by E2 and P4. Cells were obtained from cows at day 1 to 3 of the estrous cycle and plated onto either plastic culture dishes or Millicell inserts to allow basal-apical polarization. The cells were cultured with medium alone (RPMI medium + 5% charcoal-dextran stripped new-born calf serum) or with E2 (1 ng/ml), P4 (10 ng/ml) and E2+P4. The cells were then cultured for 24 h with ^{35}S methionine in the presence or absence of rbIFN-τ. At the end of culture, radiolabelled proteins secreted into the medium were examined by 2D-SDS PAGE analysis and fluorography. The result showed that two major protein spots were induced by IFN-τ. One has a molecular weight of approximately 12 kDa and pI of 6.7 (P12). This protein has been identified as macrophage migration inhibitory factor. Another induced protein has a MW of 76 kDa and pI of 4.8 (P76). In the epithelial cells, E2 decreased the secretion of protein P12 and P76 by 44% ($P < 0.05$) and 36% ($P = 0.08$) respectively, however, P4 increased the secretion of protein P76 by 59% ($P < 0.01$) after 24 h stimulation with brIFN-τ. In stromal cells, IFN-τ altered the secretion of several proteins but this was not affected by P4 or E2. Analysis of the vectorial

secretion of proteins from epithelial cells grown on Millicell inserts revealed that P12 is preferentially secreted from the apical surface ($P < 0.001$) and P76 from the basal surface ($P < 0.05$). However, the direction of secretion was not altered by steroid hormones. These data show that P12 and P76 secretion from endometrial epithelial cells were stimulated by IFN-τ and this was altered by E2 and P4. The regulation of P12 and P76 protein secretion by IFN-τ, E2 and P4 may have important implications to establishing early pregnancy and implantation in cows.

4.2. INTRODUCTION

The uterus is responsive to the continually changing endocrine status of its environment during the estrous cycle and to paracrine regulation by embryonic factors in early pregnancy [1]. Within the uterus, the steroid hormone E2 and P4 play pivotal roles in the establishment of a suitable environment for embryo implantation and pregnancy. More specifically, these steroids regulate a multitude of cellular processes during luteolysis and the initiation, maintenance and termination of pregnancy in cattle. Thus it is not surprising that these substances should participate in the regulation of synthesis and secretion of proteins which include growth factors, cytokines, extracellular matrix proteins and adhesion molecules [2, 3] in endometrium. Uterine protein expression appears to be regulated by steroids in species-specific fashion. For example, *in vivo* studies in the cycling sheep uterus suggest that expression of the tissue inhibitors of metalloproteinase-1 (TIMP-1) is down-regulated by estrogen while that of TIMP-2 may be up-regulated by progesterone [4]. In contrast, in non-human primates, progesterone withdrawal [5, 6] is associated with a rapid increase in uterine TIMP-1

expression followed by a reduction in expression. The precise mechanisms by which these steroid hormones influence protein formation and function are, however, not fully elucidated. With regard to progesterone, growth and development of the embryo and fetus are unaffected over a wide range of progesterone concentrations in the maternal plasma. Neither administration of progesterone to raise maternal concentration to about 5-fold average levels [7] or reduction of levels to about 20% by partial lutelectomy [8] affect embryonic survival or fetal and placental growth. Effects of progesterone are mediated via the nuclear progesterone receptor, a typical three-domain steroid receptor, existing in a long form (B) and two truncated forms (A and C) and acting as a transcription factor upon ligand activation [9, 10].

Pregnancy is established in cows through the release of IFN-τ by the conceptus and the inhibitory actions of this cytokine on uterine endometrial prostaglandin $F_{2\alpha}$. Interferon-tau is involved in establishment of early pregnancy in ruminants and it is released by the bovine conceptus as early as day 9 of pregnancy [11]. Interferon-tau attenuates the release of $PGF_{2\alpha}$ and indirectly rescues the corpus luteum from regression [12]. Although the inhibitory effect on gene expression has been suggested as a general feature of IFNs, the production of at least 11 proteins in the endometrium is increased by IFN-τ. 2',5' -oligoadenylate (2-5[A]) synthetase is involved in cell division and selective degradation of mRNA [13, 14]. In the IFN-induced antiviral state, this enzyme catalyzes the production of oligomers of adenine which activate a latent endoribonuclease to degrade invading viral RNA, as well as cellular RNA [15].

IFN-τ stimulates uterine secretion of an 8-kDa bovine granulocyte chemotactic protein-2 (bGCP-2). Bovine GCP-2 is an alpha-chemokine that acts primarily as a

potent chemoattractant for granulocyte cells of the immune system. bGCP-2 was not released by endometrium from day 14 nonpregnant cows, but was released in response to 25 nM IFN-τ. The regulation of bGCP-2 by IFN-τ may have important implications for cytokine networking in the uterus during pregnancy. Also, the regulation of inflammation and angiogenesis by bGCP-2 working together with other cytokines may be integral to establishing early pregnancy and implantation in the cow [16].

Bovine ubiquitin cross-reactive protein (boUCRP) is secreted by the endometrium from days 15 to 26 of pregnancy in response to IFN-τ. The transcription of the UCRP gene is transient during early pregnancy and regulated by IFN-τ [17]. The gene encoding boUCRP is under transcriptional control by the conceptus and IFN-τ. A single UCRP transcript of approximately 700 bp is present in endometrial cells cultured with 25 nM rbIFN-τ. The UCRP mRNA is not detected in endometrium on days 15, 17, 18 or 19 of the estrous cycle or in spleen, kidney, liver, corpus luteum or muscle. However, it is detectable in endometrium from pregnant cows by day 15, reaches highest levels by day 17, remained elevated on days 18, 19 and 21, and then declined to not detectable levels on day 26 [18]. UCRP, in response to rbIFN-τ becomes conjugated to endometrial cytosolic proteins during early pregnancy. The regulation of uterine proteins by UCRP may be integral to the maintenance of early pregnancy in ruminants [19].

These uterine proteins above probably mediate distal responses to IFN-τ in endometrium during early pregnancy. Because IFN-τ is produced by the blastocyst, and its mRNA is transcribed and expressed in blastocyst cells as they form on days 12-25 of pregnancy [20], it is hypothesized that IFN-τ may induce uterine proteins that are

regulated by steroid hormones in the establishment of pregnancy. These trophoblast-induced uterine proteins may not only be involved in the establishment of pregnancy but could serve, it secreted in sufficient amounts in the peripheral blood stream, as valid markers to confirm early pregnacy. Our study showed that IFN-tau induced the release of specific proteins by cultured endometrial cells [21]. The regulation of protein synthesis and secretion in bovine endometrial cells by steroid hormones are not well understood. However, sex steroid hormones are known to play an important role in the regulation of endometrial protein synthesis and secretion during pregnancy [22, 23]. The objectives of the present experiments were to determine if protein secretion induced by IFN-tau in cultured bovine epithelial and stromal cells is influenced by E2 and P4.

4.3. MATERIALS AND METHODS

4.3.1. *Chemicals and reagents*

Tissue culture medium (RPMI 1640), Hank's Buffered Saline Solution (HBSS, calcium and magnesium free), new-born calf serum (NBCS), trypan blue were purchased from GIBCO (Grand Island, NY, USA). Collagenase (Type II), trypsin (Type III, from bovine pancreas), DNase I (Type I, from bovine pancreas), Gentamicin, bovine serum albumin (BSA), 17β-estradiol and progesterone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions of estradiol and progesterone were prepared by dissolving the steroids in ethanol. Recombinant bovine interferon-τ was generously provided by Dr. R.M. Roberts (Roberts RM et al 1992). L-^{35}S-methionine was purchased from NEN (Boston, USA). Matri-gel was obtained from VWR Canlab (Ontario, Canada). Bio-Rad protein assay-dye reagent, SDS-PAGE

standards, and prestained SDS-PAGE standards were obtained from Bio-Rad Laboratories (CA, USA). ExelGel®SDS, ExelGel SDS buffer strips and Immobiline ™ DryStrip were purchased from Amersham Pharmacia BioTech AB(Sweden). Centrifugal concentrators were purchased from Pall Filtron Corporation. Kodak film X-OMAT AR was obtained from Eastman Kodak Company (Rochester, NY).

4.3.2. *Preparation of cells*

The epithelial cells were prepared as previously described [24]. Uteri from cows at days 1 to 3 of the estrous cycle (ovaries with a corpus haemorrhagicum) were collected at the slaughterhouse and transported on ice to the laboratory. Cells prepared from endometrium at this stage respond to IFN-τ in a physiological manner, that is IFN-τ inhibits the oxytocin stimulation of prostaglandin F2α secretion [25]. Briefly, the two horns of the uteri were placed in sterile HBSS containing 100 units penicillin, 100 μg streptomycin and 0.25 μg amphotericin ml^{-1}. The myometrial layers were dissected from the two horns, and the horns were then everted to expose the epithelium. The everted horns were digested for 2 hr in HBSS with 0.3% (w/v) trypsin at 37°C to obtain epithelial cells. At the end of incubation, the digested horns were scraped lightly with forceps, washed twice in HBSS and then further digested to obtain stromal cells by incubating in HBSS with 0.016% (w/v) trypsin III, 0.016% (w/v) collagenase II and 0.008% (w/v) DNase I for 45 mins at 37°C. Immediately after each cell suspension was collected, 10% NBCS was added to inhibit the trypsin.

For epithelial cells, the cell suspension was centrifuged at 60 × g for 5 min and then the pellet was washed 3 more times with HBSS. For further purification, the

epithelial cell pellet was suspended in 20 ml RPMI-1640 medium supplemented with 5% NBCS and 50 mg/ml of gentamicin and plated onto 100 x 20 mm Nunclon petri dishes (Grand Island, NY, USA) and incubated at 37°C with 5% CO_2, 95% air for 3 h. At the end of incubation, contaminating stromal cells adhered to the dish and the floating epithelial cells were collected. After cell counting and viability determination by trypan-blue exclusion, viable cells were plated onto Matrigel-coated culture dishes. 100 µl of 11% Matrigel was added to each well of 24-well plates and 200 µl was added to each well of 6-well plates and the plates were dried overnight. For stromal cells, the cell suspension was centrifuged at 60 × g for 5 min to remove clumps of cells and then the supernatant was centrifuged at 1000 × g for 10 min. The pelleted cells were washed twice with HBSS. The stromal cell suspension was plated onto dishes at a concentration of 1×10^7 cells per dish and after a 3 h incubation, the floating cells were washed away by gentle pipeting.

4.3.3. *Bovine embryo collection and dissection*

Normally cycling holstein cows were injected every 12 h for 5 days with decreasing doses of FSH (Folltropin®-V, BIONICHE Animal Health Canada Inc, ON, Canada) starting on Day 10 of the estrous cycle. On the fourth day of FSH treatment, cows received 2 injections of prostaglandin 2α (Estrumate®, Schering Canada Inc, PQ, Canada) 12 h apart. Cows were inseminated 48 h after the first prostaglandin injection. Embryos were collected on Day 16 after insemination according to standard procedures. Briefly, each uterine horn was flushed with a PBS-BSA solution several times through a cuffed Foley catheder. Flushed embryos were elonged measuring about 6-8 cm and had

a diameter of about 0.5 cm. Embryos were washed several times in PBS, and then transported to the laboratory at room temperature. Embryos were washed with RPMI-medium containing 10% charcoal free calf serum and 50 µl/ml gentamycin three times and were then dissected with a scalpel blade into 1 mm-pieces and 0.5 cm pieces.

4.3.4. *Co-culture of dissected Day 16-bovine embryos with bovine uterine epithelial cells*

In order to determine the effect of embryos, INF-τ and embryo conditioned medium on protein secretion, we performed the co-culture of embryo pieces, IFN-τ and embryo conditioned medium with bovine uterine epithelial cells. For the co-culture with embryos uterine epithelial cell cultures had been prepared as described above. Cells had been grown for 15 days in 24-well Nunclon culture dishes on Matrigel to confluence. Each well contained 1 ml RPMI-medium supplemented with 5% or 10% charcoal treated calf serum and 100 ng/ml progesterone. Culture medium had been changed every two days. On the day of embryo collection, the culture medium of epithelial cells was removed, a millicell was inserted into each of 4 culture wells and RPMI-medium with 10% charcoal treated calf serum containing 1 mm-pieces of dissected embryos were added to the millicell insert prevented the attachment between embryos and epithelial monolayers but allowed for secretions of the embryos and epithelial cells to be exchanged. One well without embryos served as control and another well was treated with recombinant bovine interferon-tau (100 ng/ml). Control medium, embryo-containing millicells and interferon treatment was left with the epithelial cells in the incubator for 24 h prior to radiolabeling. Remaining pieces of

dissected embryos were cultured in 24-well plates (seven 1 mm pieces/well in 500µl medium) and in 8-well plates (5, 10, or 15 pieces of 0.5 cm dissected embryos in 1 ml per well). The medium of these wells was changed in 24h or 48 h intervals so that 24h or 48h-conditioned medium was collected over a period of 1 week. Conditioned medium was frozen at -20°C.

To test the effect of embryo-conditioned medium on protein secretion, embryo conditioned medium (seven 1mm –pieces in 500 µl of medium with 10% charcoal treated calf serum) was separated from embryo tissue by centrifugation and used as culture medium for 24 h on epithelial cells (16 days in culture). After 24h, radio-labeling was performed for 24 h as described below.

4.3.5. *Hormone treatment of cell cultures*

Epithelial and stromal cells were cultured for 8 days in PRMI-medium containing 10% charcoal treated calf serum. Then cells were treated without steroids (control), with E2 (10ng/ml), P4 (100ng/ml) or combination of E2 and P4 until Day 14. Then medium was removed and cells were incubated for 24 h in the presence or absence of IFN-τ, IFN-τ + E2, IFN-τ + P4, IFN-τ + E2+P4 for 24 h. Medium from these 24 h incubations were harvested and frozen at -20°C until 2D-PAGE analysis.

4.3.6. *Radioactive labeling of Secreted Proteins and 2D-PAGE*

The confluent epithelial cells were incubated in the presence or absence of IFN-τ, IFN-τ + E2, IFN-τ + P4, IFN-τ + E2+P4 for 24 h at 37°C. Cells were washed and incubated with methionine-free RPMI-1640 medium for 30 min. The medium was then

replaced with 500 µl of methionine-free RPMI-1640 medium containing 5 µl of ^{35}S-labeled methionine (50 µCi) and the cells were incubated in the presence or absence of 100 ng/ml IFN-τ for a further 24 h. The medium was removed and stored at -70 °C until protein extraction.

Before separation and analysis of the proteins by means of two-dimensional SDS PAGE (2-D PAGE), the culture medium was centrifuged (500 × g for 10 min) to remove cell debris prior to protein extraction. The proteins were concentrated to 50 µl using Ultrafree-15 concentrators (5000 MW cutoff; Millipore) and added to IPG buffer (8M Urea, 2% CHAPS, 0.5% IPG Buffer (pH 3-10), bromophenol blue, 65 mM DTT). Prior to loading, a 5 µl aliquot of each sample was removed for radioactive counts then 5 µl internal protein standards were added to each sample so that the molecular weight and isoelectric points (pI) of found proteins could be estimated. The separation in first dimension was carried out using Immobiline DryStrips (pH 3-10) which had been rehydrated for at least 10 hours in an Immobiline™ Drystrip Reswelling Tray. The samples were then separated on a MultiPhore II flatbed system for 16 hrs at 15°C. The voltage was 300 V for the first 3h, from 300 to 2000 V during the following 5 h, and finally 8 h at 2000 V. Before the second dimension was performed, the dry strips were first equilibrated for 10 min in Equilibration solution 1 (0.5 M Tris/HCl pH 6.8, 3.6 g urea, 3 ml glycerol, 0.1 g SDS, 25 mg DDT and distilled water up to 10 ml) and another 10 min in Equilibration solution 2 (0.5 M Tris/HCl pH 6.8, 3.6 g urea, 3 ml glycerol, 0.1 g SDS, 0.45g iodoacetamide and distilled water up to 10 ml). The second dimension was performed after placing the strips on Pharmacia ExcelGel® XL SDS 8-18 using the MultiPhore II flatbed system at 15°C. After running the gels, they were immediately

immersed in fixing solution (50% methanol, 10% acetic acid in water) and stained with Coomassie blue, destained, incubated in a radiographic enhancer and then in a preserving solution. The gels were wrapped in cellophane, air-dried and exposed with Kodak radiographic film for various times. Protein spots on control and IFN-τ gels were compared, and molecular weight and pI estimated using the computer program Phoretix 2D (Version 4.00, Nonlinear Dynamics Ltd, UK).

4.3.7. Analysis of vectorial secretion of proteins from epithelial cells

A millicell was inserted into each of 4 culture wells, then 100 µl of 11% Matrigel was added to each well of 4-well plates onto millicell insert and dried overnight. After cell counting and viability determination by trypan-blue exclusion, viable cells were plated onto Matrigel-coated millicell inserts. The epithelial cells were grown on a matrigel matrix Millicell insert. The cells retain a three dimensional structure and become spatially oriented such that that apical and basal secretion can be monitored separately. The medium inside and outside of millicells were collected and were subject to 2D-SDS PAGE analysis.

4.3.8. *Statistical analysis*

Each experiment was carried out using the cells from one uterus and was repeated with three different uteri. For protein secretion, the data were analyzed using model y = EXP + TREATMENT (EXP) + RESIDUAL. Differences between individual means were determined by student test. A probability of $P < 0.05$ was considered to be

statistically significant. The data were analyzed using the computer program SAS (SAS Institute Inc., Cary, NC).

4.4. RESULTS

4.4.1. *Effects of embryo, conditioned medium and IFN-τ on protein secretion in bovine endometrial epithelial cells*

In order to determine the effect of embryo, conditioned medium and IFN-τ on protein secretion, the confluent cultures of bovine endometrial epithelial cells were treated with or without embryo, conditioned medium and IFN-τ and the ^{35}S-methionine labeled proteins were analyzed by 2D-PAGE. Figure 1 shows representative 2D-gel autoradiographs of labeled proteins from control and co-culture with embryo, conditioned medium and IFN-τ. A comparison of control (Fig. 1A), co-culture cells with IFN-τ (Fig. 1B), embryo (Fig. 1C) and conditioned medium (Fig. 1D) showed that two protein spots, one with the estimated pI of 4.8 and MW of 76 kDa (designated P76) and the other with a pI of 6.7 and MW of 12 kDa (designated P12), were present in co-culture with embryo, conditioned medium and IFN-τ but not in control cells.

4.4.2. *Effect of IFN-τ on proteins secreted by endometrial stromal cells*

Confluent endometrial stromal cells were cultured in absence or presence of IFN-τ (100 ng/ml) for 24 h as described in Materials and Methods. Radiolabeled medium proteins (200,000 cpm) were separated by 2 dimensional PAGE. The resulting representative autoradiographs were generated by exposure of X-ray film to the dried

gel for varied times. Figure 2 shows depicting 2D-PAGE profiles of proteins containing incorporated s^{35}-methionine and secreted by bovine stromal cells in the control (Fig 2**A**) and presence of rbIFN-τ (Fig 2**B**). The changes in protein secretion were also detectable in bovine endometrial stromal cells, but different from those observed in epithelial cells.

4.4.3. *Effects of E2 and P4 on protein secretion induced by IFN-τ in bovine uterine epithelial cells*

In order to determine the effect of E2 and P4 on protein secretion induced by IFN-τ, the confluent cultures of bovine endometrial epithelial cells were treated with or without E2, P4 and E2 + P4 in the presence and absence of IFN-τ and the ^{35}S-methionine labeled proteins were analyzed by 2D-PAGE. Figure 3A and 3B show representative 2D-gel autoradiographs of labeled proteins from control and IFN-τ, IFN-τ + E2 and IFN-τ + P4 treated epithelial cells. In the epithelial cells, E2 decreased the secretion of protein P12 and P76 by 44% ($P < 0.05$) and 36% ($P = 0.08$) respectively, however, P4 increased the secretion of protein P76 by 59% ($P < 0.01$) after 24 h stimulation with brIFN-tau. In stromal cells, IFN-tau altered the secretion of several proteins but this was not affected by P4 or E2.

4.4.4. *Analysis of the vectorial secretion of proteins from epithelial cells*

To analyze the vectorial secretion of proteins from epithelial cells, the epithelial cells were grown on a matrigel matrix Millicell insert. These autoradiograms shows the different profile of basal (Fig 4**A**) and apical (Fig 4**B**) secretion. The result revealed that

P12 is preferentially secreted from the apical surface ($P < 0.01$) and P76 from the basal surface ($P < 0.05$). However, the direction of secretion was not altered by steroid hormones.

4.5. DISCUSSION

The bovine conceptus produces IFN-tau during early stages of pregnancy (Days 12-25) [20]. This IFN-τ is a major product of the conceptus and may be involved in coordination of endocrine and/or paracrine events in the uterus to provide an environment that is favorable for maintenance of pregnancy in ruminants. The present study is the first to simultaneously examine the effects of embryo pieces, IFN-τ and embryo conditioned medium on protein secretion from bovine endometrial epithelial cells and the hormone modulation of IFN-τ induced protein secretion. Through evaluating proteins secreted by the endometrial epithelial and stromal cells in response to co-culture with embryo, conditioned medium and rbIFN-τ by 2D-PAGE in vitro, we have identified two molecular size classes of uterine proteins. One has a molecular weight of approximately 12 kDa and pI of 6.7 (P12). This protein has been identified as macrophage migration inhibitory factor [21]. Another induced protein has a MW of 76 kDa and pI of 4.8 (P76). These experiments confirm that a recombinant IFN-τ, embryos and embryos conditioned medium can induce several protein synthesis and secretion by endometrial epithelial and stromal cells derived from cows. These proteins provide a sensitive and useful marker for IFN-τ action on uterine endometrial cells.

Progesterone is secreted by the corpus luteum and by the placenta and is responsible for preparing the body for pregnancy and, if pregnancy occurs, maintaining

it until birth. Estrogens are a family of structurally related hormones which play a role in many different tissue types, affecting both female and male physiology. In some tissues, particularly those associated with reproductive function, estrogens are a driver of tissue development and activity [26]. After the E2 and P4 treatment of the endometrial epithelial cells, E2 decreased the secretion of protein P12 and P76 by 44% ($P < 0.05$) and 36% ($p < 0.08$) respectively, whereas P4 increased the secretion of protein p76 by 59% ($P < 0.01$) after 24 h stimulation with brIFN-τ. In stromal cells, IFN-τ altered the secretion of several proteins but this was not affected by P4 or E2. The results of the present study indicated that exposure to progesterone enhanced responses of endometrial epithelial cells to IFN-τ. Based on these observations, we speculate the increased circulatory levels of E2 and/or a fall in P4 at term may be responsible for reduced specific protein synthesis and secretion in endometrium.

It is well known that under the influence of ovarian hormones, the rabbit uterus undergoes marked biochemical [27] morphological changes [28]. These transformations are believed to be associated with the preparation of a receptive endometrium, essential for the implantation of the blastocyst. Our results show that after treatment with steroid hormones changes in protein secretion occurred in endometrial epithelial and stromal cell providing evidence that the protein secretory activity of the bovine endometrium is under the control of P4 and E2.

A critical factor in the expression of secretory protein function is mode of localization. Secretory protein pathways of epithelial layers have been segregated into apical or basolateral domains [29]. Results of experiments using vectorial secretion analysis of proteins from epithelial cells indicate that protein secretion is directed in

different profile of basal and apical secretion. P12 secretion is preferentially directed in an apical orientation and P76 a basal orientation. However, the direction of secretion was not altered by steroid hormones. This is generally consistent with our previous report [21] that P12 was identified as MIF, MIF staining was particularly intense on the apical side of the luminal epithelium and in superficial glandular endometrium. However other work [30] has shown that PGF2a is preferentially secreted in the basal compared to the apical compartment of endometrial epithelial cells and the direction of secretion is about influenced by steroid hormone treatment.

To summarize, our results show that E2 decreases the IFN-τ-stimulated secretion of P12 and P76, while progesterone increases P12 and P76 secretion. The differences seen under different hormone conditions in P12, P76 secretion responses in the present study make this a very useful system to identify the influence of sex hormone during early stage of pregnancy. These findings have important implications for future early pregnancy diagnosis. The regulation of P12 and P76 protein secretion by IFN-τ, E2 and P4 may have important implications to establishing early pregnancy and implantation in cows.

4.6. Acknowledgment

We thank Mira Dobias-Goff and Daniell Rannou for technical assistance and Dr Yule Pan for statistical assistance.

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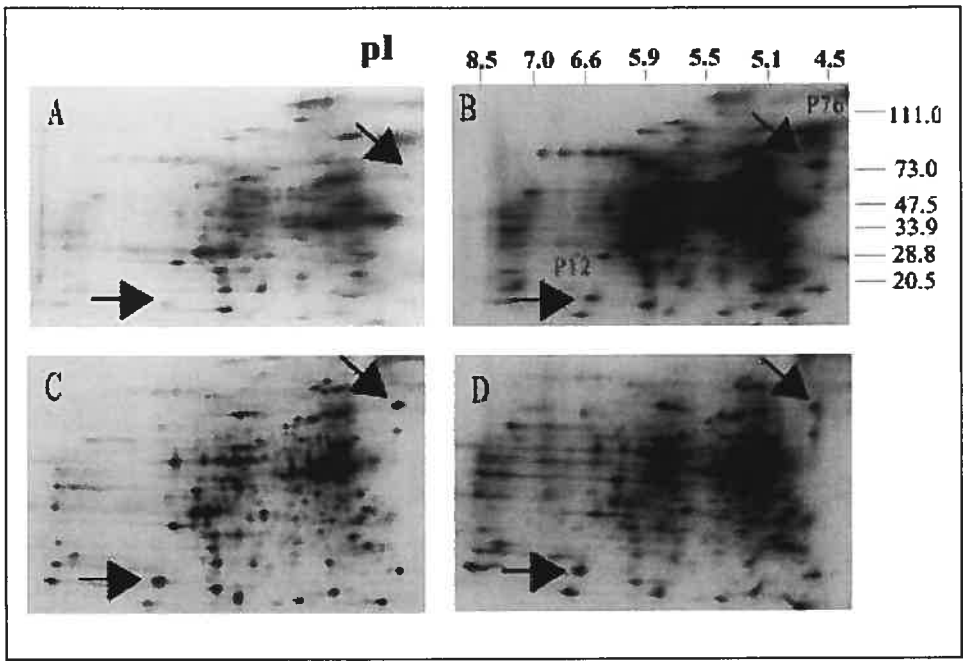

Figure 1. Effect of Embryo, conditioned medium and IFN-τ on proteins secreted by endometrial epithelial cells. Cells were cultured in the absence or presence of embryo, conditioned medium and IFN-t (100 ng/ml) for 24 h as described in Materials and Methods. Radiolabeled medium proteins (200,000 cpm) in the culture medium were separated by 2 dimensional PAGE. The resulting representative autoradiographs were generated by exposure of X-ray film to the dried gel for varied times, depicting 2D-PAGE profiles of proteins containing incorporated s^{35}-methionine and secreted by bovine epithelial cells. (A) control, (B) rbIFN-τ, (C) conditioned medium and (D) presence of embryo .

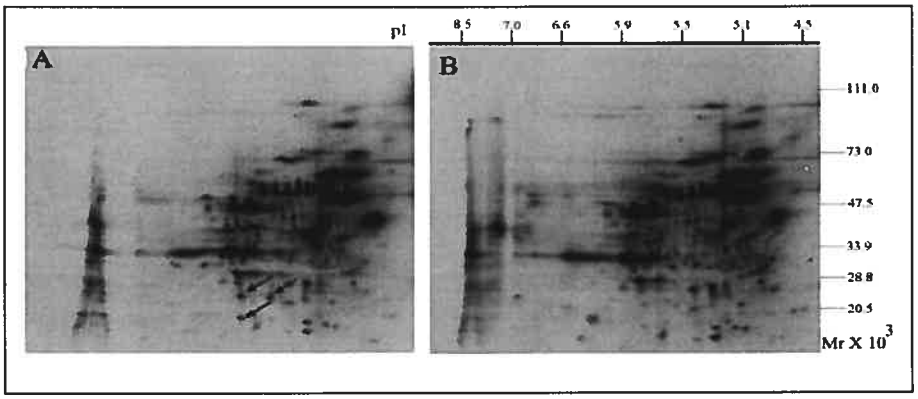

Figure 2. Effect of IFN-τ on proteins secreted by endometrial stromal cells. Confluent cells were cultured in the absence or presence of IFN-τ (100 ng/ml) for 24 h as described in Materials and Methods. Radiolabeled medium proteins (200,000 cpm) in the culture medium were separated by 2 dimensional PAGE. The resulting representative autoradiographs were generated by exposure of X-ray film to the dried gel for varied times, and depicting 2D-PAGE profiles of proteins containing incorporated s^{35}-methionine and secreted by control (**A**) and presence of rbIFN-τ (**B**). Changes in protein secretion were detectable between control and rbIFN-τ– treated cells, but were different from those observed in epithelial cells.

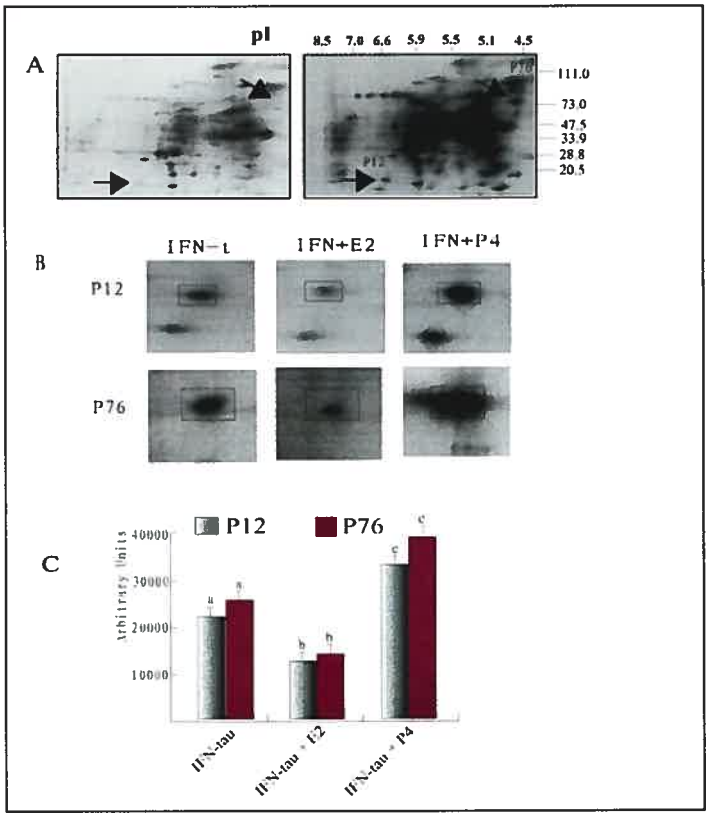

Figure 3. Influence of E2 and P4 on protein secretion induced by IFN-τ in bovine epithelial cells. Cells were cultured to confluence in absence or presence of E2 and P4, the cells were then cultured for a further 24 h in absence or presence of IFN-τ (100 ng/ml) as described in Materials and Methods. **A**. Representative autoradiograph of 2D-PAGE separated proteins from the culture medium of bovine epithelial cells, the left panel is control and the right panel is IFN-τ treated. **B**. Enlargements of the P12 and P76 proteins from gels loaded with proteins from treatment IFN-τ alone, IFN-τ + E2 and IFN-τ + P4. **C**. Graph shows the quantitative measurement of spot density of IFN-τ, IFN-τ + E2, and IFN-τ + P4 treatment. Data are expressed as the least-square means ± SEM (n = 3).

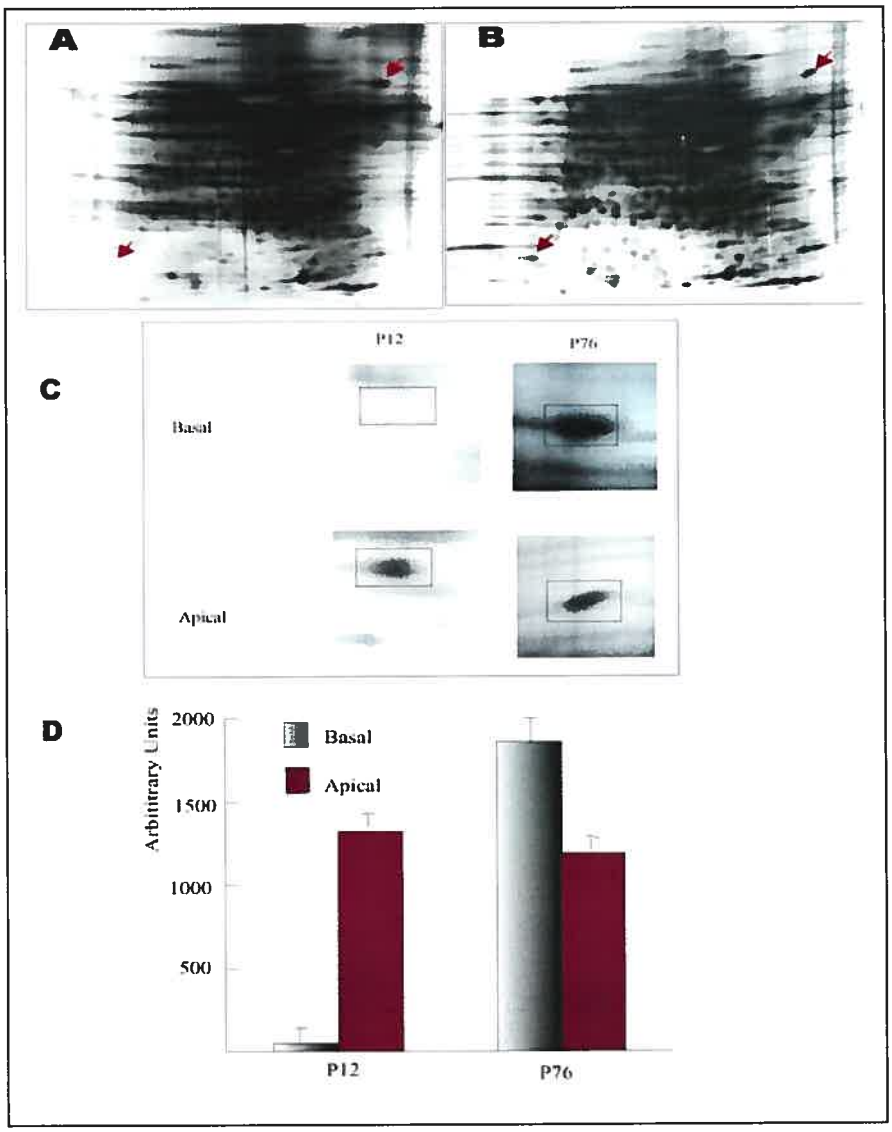

Figure 4. The Epithelial Cells were grown on a matrigel matrix Millicell insert. The cells retain a three dimensional structure and become spatially oriented such that that apical and basal secretion can be monitored separately. These autograms shows the different profile of basal (**A**) and apical (**B**) secretion. (**C**) Enlargement of the P12 and P76 protein sports denoted by arrows in **Fig. 4A and B**. (**D**) Graph shows the quantitative measurement of P12 and P76 protein spot density in basal and apical gel. Data are expressed as the least-square means ± SEM (n = 3).

5. Article Two

Objective

To identify and characterize specific proteins secreted from endometrial epithelial cells in response to IFN-t that could be important for endometrial function and/or embryo development.

BIOLOGY OF REPRODUCTION 69, 1690–1696 (2003)
Published online before print 9 July 2003.
DOI 10.1095/biolreprod.102.012559

Interferon-τ Stimulates Secretion of Macrophage Migration Inhibitory Factor from Bovine Endometrial Epithelial Cells[1]

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ABSTRACT

During early pregnancy in ruminants, the embryo not only prevents prostaglandin $F_{2\alpha}$ release, but it also modifies protein synthesis in the endometrium. This is accomplished by the secretion of interferon-tau (IFN-τ) from the embryo. The objective of this study was to identify and characterize specific proteins secreted from endometrial epithelial cells in response to IFN-τ that could be important for endometrial function and/or embryo development. The epithelial cells were prepared and cultured to confluence and then incubated with or without 100 ng/ml IFN-τ. At the end of the incubation, the proteins in the medium were analyzed by two-dimensional PAGE. The result showed that two major protein spots were induced by IFN-τ. One has a molecular mass of approximately 12 kDa and an isoelectric point (pI) of 6.7; the other has a molecular mass of 76 kDa and pI of 4.8. Protein sequence analysis showed that the 12-kDa protein contained a partial amino acid sequence that corresponded to macrophage migration inhibitory factor (MIF). To determine whether MIF is expressed in endometrial cells, isolated stromal or epithelial cells were incubated with or without 100 ng/ml IFN-τ for 0, 3, 6, 12, 24, and 48 h. After incubation, the MIF protein in cells was examined by Western blotting analysis, and the steady-state mRNA for MIF was examined by Northern analysis. Results showed that MIF protein and mRNA were present in the epithelial cells but not the stromal cells. The presence of MIF in the luminal epithelium of endometrial tissue was confirmed by immunohistochemistry. However, there was no effect of IFN-τ on MIF expression in the epithelial cells. The concentration of MIF in the medium was quantified by Western blotting analysis to determine if IFN-τ altered MIF protein secretion from the epithelial cells. The results showed that IFN-τ significantly stimulated the secretion of MIF protein from the cells. These data show that MIF is expressed in the epithelial, but not the stromal, cells of the endometrium and that MIF secretion from the epithelial cells is stimulated by IFN-τ. It is therefore likely that MIF plays a role in early embryo development, and further characterization of MIF expression and its regulation in the endometrium will add significantly to our understanding of early embryo-uterine interactions.

conceptus, embryo, female reproductive tract, pregnancy, uterus

INTRODUCTION

During early pregnancy in ruminants, the embryo not only prevents prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release, but it also modifies protein secretion from the endometrium [1, 2]. Type I trophoblast interferon-tau (IFN-τ) is the antiluteolytic protein secreted by conceptuses of ruminants during maternal recognition of pregnancy. IFN-τ is secreted specifically by the conceptus and is released by the bovine conceptus as early as Day 9 of pregnancy [2]. Secretion of IFN-τ increases until about Day 17 when it attenuates the release of $PGF_{2\alpha}$ and indirectly rescues the corpus luteum from regression [3] and then secretion declines. Cytokines appear to play critical roles in the establishment of pregnancy and IFN-τ, apart from preventing the luteolytic secretion of $PGF_{2\alpha}$, also induces the alpha chemokine, granulocyte chemotactic protein-2 [4, 5], and several other uterine proteins such as 2′,5′-oligoadenylate synthetase [6], acidic secretory protein [7], and ubiquitin cross-reactive protein [8, 9]. These uterine proteins probably mediate distal responses to IFN-τ in the endometrium during early pregnancy. The exact role that these proteins play in early pregnancy is still not fully understood, but they are probably involved in growth and development of the embryo, remodeling of the endometrium during attachment, and modulating the immune response.

Under normal conditions, the conceptus (the fetus and its membranes) expresses paternal antigens but is not rejected by the maternal immune system. The conceptus escapes destruction by immune effector cells by expressing atypical major histocompatibility complex molecules [10, 11], which protect the invasive trophoblast against attack by cytotoxic lymphocytes [12] and natural killer (NK) cells [13], and by producing factors that stimulate the production of beneficial cytokines [14, 15]. Few studies have concentrated on immunomodulatory events during the peri-implantation period in ruminants[16] but there is evidence that local immunity plays an important role in the fate of the ruminant conceptus. Immune cells have been reported in bovine [17] and ovine uteri during pregnancy [17, 18], and lymphokine-activated killer cells exert lytic damage on preattachment conceptuses [19]. Furthermore, uterine milk proteins, which are secreted by the endometrium of ruminants in response to IFN-τ, can inhibit NK cells [20].

Because the embryo interacts predominantly with the epithelial cells of the endometrium, the objectives of this study were: 1) to use high resolution two-dimensional (2D) electrophoresis to characterize the IFN-τ-induced changes in the secretion of newly synthesized proteins from isolated endometrial epithelial cells and 2) to identify specific proteins induced by IFN-τ that are involved in embryo-uterine interactions during early pregnancy.

MATERIALS AND METHODS

Chemicals and Reagents

Cell culture medium (RPMI 1640), Hanks buffered saline solution (HBSS, calcium and magnesium free), newborn calf serum (NBCS), an-

[1]This work was supported by grants from NSERC (to A.K.G.).
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Received: 21 October 2002.
First decision: 12 November 2002.
Accepted: 11 June 2003.

ISSN: 0006-3363. http://www.biolreprod.org

Interferon-tau Stimulates Secretion of Macrophage Migration Inhibitory Factor from Bovine Endometrial Epithelial Cells[1]

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Key words: Bovine, uterus, endometrial cells, interferon-tau, MIF

[1] This work was supported by grants from NSERC (AKG)

5.1. Abstract

During early pregnancy in ruminants, the embryo not only prevents prostaglandin $F_{2\alpha}$ release, but it also modifies protein synthesis in the endometrium. This is accomplished by the secretion of interferon-tau (IFN-τ) from the embryo. The objective of this study was to identify and characterize specific proteins secreted from endometrial epithelial cells in response to IFN-τ that could be important for endometrial function and/or embryo development. The epithelial cells were prepared and cultured to confluence and then incubated with or without 100 ng/ml IFN-τ. At the end of the incubation, the proteins in the medium were analyzed by two-dimensional PAGE. The result showed that two major protein spots were induced by IFN-τ. One has a molecular mass of approximately 12 kDa and an isoelectric point (pI) of 6.7; the other has a molecular mass of 76 kDa and pI of 4.8. Protein sequence analysis showed that the 12-kDa protein contained a partial amino acid sequence that corresponded to macrophage migration inhibitory factor (MIF). To determine whether MIF is expressed in endometrial cells, isolated stromal or epithelial cells were incubated with or without 100 ng/ml IFN-τ for 0, 3, 6, 12, 24, and 48 h. After incubation, the MIF protein in cells was examined by Western blotting analysis, and the steady-state mRNA for MIF was examined by Northern analysis. Results showed that MIF protein and mRNA were present in the epithelial cells but not the stromal cells. The presence of MIF in the luminal epithelium of endometrial tissue was confirmed by immunohistochemistry. However, there was no effect of IFN-τ on MIF expression in the epithelial cells. The concentration of MIF in the medium was quantified by Western blotting analysis to determine if IFN-τ altered MIF protein secretion from the epithelial cells. The results showed that IFN-τ significantly

stimulated the secretion of MIF protein from the cells. These data show that MIF is expressed in the epithelial, but not the stromal, cells of the endometrium and that MIF secretion from the epithelial cells is stimulated by IFN-τ. It is therefore likely that MIF plays a role in early embryo development, and further characterization of MIF expression and its regulation in the endometrium will add significantly to our understanding of early embryo-uterine interactions.

5.2. Introduction

During early pregnancy in ruminants, the embryo not only prevents prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release, but it also modifies protein secretion from the endometrium [1, 2]. Type I trophoblast interferon-tau (IFN-τ) is the antiluteolytic protein secreted by conceptuses of ruminants during maternal recognition of pregnancy. IFN-τ is secreted specifically by the conceptus and is released by the bovine conceptus as early as Day 9 of pregnancy [2]. Secretion of IFN-τ increases until about Day 17 when it attenuates the release of $PGF_{2\alpha}$ and indirectly rescues the corpus luteum from regression [3] and then secretion declines. Cytokines appear to play critical roles in the establishment of pregnancy and IFN-τ, apart from preventing the luteolytic secretion of $PGF_{2\alpha}$, also induces the alpha chemokine, granulocyte chemotactic protein-2 [4, 5], and several other uterine proteins such as 2',5'-oligoadenylate synthetase [6], acidic secretory protein [7], and ubiquitin cross-reactive protein [8, 9]. These uterine proteins probably mediate distal responses to IFN-τ in the endometrium during early pregnancy. The exact role that these proteins play in early pregnancy is still not fully understood, but they are probably involved in growth and development of the embryo, remodeling of the endometrium during attachment, and modulating the immune response.

Under normal conditions, the conceptus (the fetus and its membranes) expresses paternal antigens but is not rejected by the maternal immune system. The conceptus escapes destruction by immune effector cells by expressing atypical major histocompatibility complex molecules [10, 11], which protect the invasive trophoblast against attack by cytotoxic lymphocytes [12] and natural killer (NK) cells [13], and by producing factors that stimulate the production of beneficial cytokines [14, 15]. Few studies have concentrated on immunomodulatory events during the peri-implantation period in ruminants[16] but there is evidence that local immunity plays an important role in the fate of the ruminant conceptus. Immune cells have been reported in bovine [17] and ovine uteri during pregnancy [17, 18], and lymphokine-activated killer cells exert lytic damage on preattachment conceptuses [19]. Furthermore, uterine milk proteins, which are secreted by the endometrium of ruminants in response to IFN-τ, can inhibit NK cells [20].

Because the embryo interacts predominantly with the epithelial cells of the endometrium, the objectives of this study were: 1) to use high resolution two-dimensional (2D) electrophoresis to characterize the IFN-τ-induced changes in the secretion of newly synthesized proteins from isolated endometrial epithelial cells and 2) to identify specific proteins induced by IFN-τ that are involved in embryo-uterine interactions during early pregnancy.

5.3. MATERIALS AND METHODS

5.3.1. Chemicals and Reagents

Cell culture medium (RPMI 1640), Hanks buffered saline solution (HBSS, calcium and magnesium free), newborn calf serum (NBCS), antibiotics, and trypan blue were purchased from Gibco (Grand Island, NY). Collagenase (type II), trypsin

(type III, from bovine pancreas), DNase I (type I, from bovine pancreas), gentamicin, BSA, and progesterone were purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution of progesterone was prepared by dissolving the steroid in ethanol. Matrigel was obtained from VWR Canlab (Montreal, QC, Canada). Protein assay-dye reagent concentrate and electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). Mouse anti-human migration inhibitory factor (MIF) antibody and mouse IgG were purchased from Cedarlane Laboratories Limited (Hornby, ON, Canada). Biotrans nylon membranes (0.2 μm) were obtained from ICN Pharmaceuticals (Montreal, PQ, Canada). [α-^{32}P]dCTP and [^{35}S]ATP were obtained from Mandel Scientific NEN Life Science Products (Mississauga, ON, Canada). RNA ladder (0.24–9.5 kb), 1 kb DNA ladder, and tissue culture plates were obtained from Corning-Costar (Fisher Scientific, Montreal, PQ, Canada). X-OMAT AR film was obtained from Eastman Kodak Company (Rochester, NY). The recombinant bovine IFN-τ was a generous gift from Dr. R. Michael Roberts (University of Missouri). The MIF cDNA was a generous gift from Dr. A. Meinhardt (Philipps-University, Marburg, Germany).

5.3.2. Preparation and Culture of Cells

The epithelial cells were prepared as previously described [21]. Uteri from cows at Days 1–3 of the estrous cycle (ovaries with a corpus hemorrhagicum) were collected at the slaughterhouse and transported on ice to the laboratory. Cells prepared from endometrium at this stage respond to IFN-τ in a physiological manner, i.e., IFN-τ inhibits the oxytocin stimulation of $PGF_{2\alpha}$ secretion [22]. Briefly, the two horns of the uteri were placed in sterile HBSS containing 100 U penicillin, 100 μg streptomycin, and 0.25 μg amphotericin ml^{-1}. The myometrial layers were dissected

from the two horns, and the horns were then everted to expose the epithelium. The everted horns were digested for 2 h in HBSS with 0.3% (w/v) trypsin at 37°C to obtain epithelial cells. At the end of incubation, the digested horns were scraped lightly with forceps, washed twice in HBSS, and then further digested to obtain stromal cells by incubating in HBSS with 0.016% (w/v) trypsin III, 0.016% (w/v) collagenase II, and 0.008% (w/v) DNase I for 45 min at 37°C. Immediately after each cell suspension was collected, 10% NBCS was added to inhibit the trypsin.

For epithelial cells, the cell suspension was centrifuged at 60 x *g* for 5 min and then the pellet was washed 3 more times with HBSS. For further purification, the epithelial cell pellet was suspended in 20 ml RPMI-1640 medium supplemented with 5% NBCS and 50 mg/ml of gentamicin and plated onto 100 x 20 mm Nunclon Petri dishes (Grand Island, NY) and incubated at 37°C with 5% CO_2, 95% air for 3 h. At the end of incubation, contaminating stromal cells adhered to the dish and the floating epithelial cells were collected. After cell counting and viability determination by trypan-blue exclusion, viable cells were plated onto Matrigel-coated culture dishes. One hundred microliters of 11% Matrigel were added to each well of 24-well plates and 200 µl was added to each well of 6-well plates and the plates were dried overnight. For stromal cells, the cell suspension was centrifuged at 60 x *g* for 5 min to remove clumps of cells and then the supernatant was centrifuged at 1000 x *g* for 10 min. The pelleted cells were washed twice with HBSS. The stromal cell suspension was plated onto dishes at a concentration of 1 x 10^7 cells per dish, and after a 3-h incubation, the floating cells were washed away by gentle pipetting.

The cells were cultured at 37°C with 5% CO_2, 95% air until they were confluent (about 7 days) in RPMI-medium supplemented with 10% NBCS and 100

ng/ml progesterone. The culture medium was changed every 2 days. The homogeneity of the cell populations was examined by immunocytochemistry. Epithelial cell contamination of stromal cells was about 3%, and stromal cell contamination of epithelial cells was less than 1% [21].

5.3.3. Radioactive Labeling of Secreted Proteins and 2D-PAGE

The confluent epithelial cells were incubated in the presence or absence of 100 ng/ml IFN-τ for 24 h at 37°C. Cells were washed and incubated with methionine-free RPMI-1640 medium for 30 min. The medium was then replaced with 500 µl of methionine-free RPMI-1640 medium containing 5 µl (50 µCi) of ^{35}S-labeled methionine (specific activity >1200 Ci/mmol) and the cells were incubated in the presence or absence of 100 ng/ml IFN-τ for a further 24 h. The medium was removed and stored at -70°C until protein extraction.

Before separation and analysis of the proteins by means of 2D SDS-PAGE (2D PAGE), the culture medium was centrifuged (500 x *g* for 10 min) to remove cell debris prior to protein extraction. The proteins were concentrated to 50 µl using Ultrafree-15 concentrators (5000 MW cutoff; Millipore, Bedford, MA) and added to IPG buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer [pH 3–10], bromophenol blue, 65 mM dithiothreitol [DTT]). Prior to loading, a 5-µl aliquot of each sample was removed for radioactive counts, and 5 µl internal protein standards were added to each sample so that the molecular mass and isoelectric points (pI) of found proteins could be estimated. The separation in first dimension was carried out using Immobiline DryStrips (pH 3–10), which had been rehydrated for at least 10 h in an Immobilin Drystrip reswelling tray (Amersham Pharmacia Biotech AB, Baie d'Urfé, QC, Canada). The samples were then separated on a MultiPhore II flatbed system

(Amersham) for 16 h at 15°C. The voltage was 300 V for the first 3 h, from 300 to 2000 V during the following 5 h, and finally 8 h at 2000 V. Before the second dimension was performed, the dry strips were first equilibrated for 10 min in equilibration solution 1 (0.5 M Tris/HCl pH 6.8, 3.6 g urea, 3 ml glycerol, 0.1 g SDS, 25 mg DDT, and distilled water up to 10 ml) and another 10 min in equilibration solution 2 (0.5 M Tris/HCl pH 6.8, 3.6 g urea, 3 ml glycerol, 0.1 g SDS, 0.45 g iodoacetamide, and distilled water up to 10 ml). The second dimension was performed after placing the strips on Pharmacia ExcelGel XL SDS 8–18 using the MultiPhore II flatbed system at 15°C. After running the gels, they were immediately immersed in fixing solution (50% methanol, 10% acetic acid in water) and stained with Coomassie blue, destained, and incubated in a radiographic enhancer and then in a preserving solution. The gels were wrapped in cellophane, air dried, and exposed with Kodak radiographic film for various times. Protein spots on control and IFN-τ gels were compared, and molecular weight and pI estimated using the computer program Phoretix 2D (version 4.00, Nonlinear Dynamics Ltd., Newcastle upon Tyne, UK).

5.3.4. Protein Sequencing

To obtain proteins for sequencing, the procedure for culture and incubation of the cells was the same as described above except that the proteins were not labeled with ^{35}S-methionine. The 2D gels were run, each loaded with 50 µg of unlabeled proteins. After 2D PAGE the proteins were stained with silver nitrate (silver staining kit, Amersham), and the spots of interest were excised. The sequence analysis was performed at the Harvard Microchemistry Facility by Microcapillary reverse-phase

HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQDECA quadruple ion-trap mass spectrometer.

5.3.5. Isolation of Total Cellular RNA and Northern Blot Analysis

Bovine epithelial cells were cultured as described above and were then incubated with or without 100 ng of IFN-τ/ml for different times in 6-well flat-bottom plates. After the incubation the total cellular RNA was isolated according to the manufacturer's specifications (Rneasy Mini Kit (50), Qiagen, Mississauga, ON, Canada). For Northern analysis, RNA samples (10 µg) were denatured at 70°C for 5 min in denaturing buffer, electrophoresed on a 1.2% agarose gel, and transferred overnight by capillarity to a nylon membrane, as previously described [23]. The membranes were UV treated (150 mJ) and prehybridized as described by Johnson et al. [24]. A ladder of RNA standards was run with each gel, and ethidium bromide (10 µg) was added to each sample prior to electrophoresis to compare RNA loading and determine migration of standards. The membrane was first hybridized to MIF cDNA probe overnight at 42°C, which was randomly primed with 50 µCi α-^{32}P-dCTP using QuikHyb solution (Stratagene, La Jolla, CA). Blots were washed as described previously [24]. After stripping the radioactivity with 0.1% saline-sodium citrate (0.15 M NaCl and 0.015 M sodium citrate)-0.1% SDS for 30 min at 100°C, the same blot was subsequently hybridized with a pig glyceraldehyde-3-phosphate dehydrogenase. (GAPDH) cDNA as a control gene for RNA loading and transfer [25]. Probes were labeled with [α-^{32}P]deoxy-CTP using the Prime-a-Gene labeling system (Promega, Madison, WI) to a final specific activity greater than 1 x 10^8 cpm/µg DNA. The membranes were then scanned using a Storm 840

PhosphorImager scanner and quantified by densitometry using ImageQuant software (version 1.2), (both from Molecular Dynamics, Inc., Sunnyvale, CA).

5.3.6. Protein Extracts and Immunoblot Analysis

Bovine epithelial cells were cultured as described above and then incubated with or without 100 ng of IFN-τ/ml for different times in 6-well flat-bottom plates. After incubation the medium was removed and solubilized protein extracts were prepared from the cells as previously described [23], with minor modifications. Briefly, after treatment, uterine cells were rinsed with HBSS and detached from the dish with 250 µl TED sonification buffer (20 mM TRIS, 50 mM EDTA, 0.1 mM diethyldithiocarbamic acid [DEDTC], pH 8.0) containing 32 mM octyl glucoside and then sonicated (8 sec/cycle; three cycles). The sonicates were centrifuged at 13 000 x *g* for 25 min at 4°C. The supernatants (solubilized cytoplasmic and cell extracts) were stored at -70°C until immunoblotting analysis. The medium (removed as described above) was centrifuged (500 x *g* for 10 min) to remove cell debris prior to protein extraction. The proteins in the medium were concentrated using Ultrafree-15 concentrators (5000 MW cutoff; Millipore). The protein concentration was determined by the method of Bradford.

Proteins (25 µg) of cell and medium extracts were resolved by one-dimensional SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Hybond-ECL; Amersham Life Science, Inc, Buckinghamshire, UK). The blots were incubated for 18 h at 4°C in the presence of mouse anti-human MIF monoclonal immunoglobulin (Ig) G, diluted to 1:3000. Blots were washed, incubated with second antibody (anti-mouse IgG), and exposed to chemiluminescence detection substrates as described. The membranes were scanned as described above.

The molecular size of immunoreactive bands was determined by comigration of a ladder of biotinylated SDS-PAGE molecular weight standards (Bio-Rad Laboratories) applied to a lane in each gel. Prestained standards were also applied to gels to assess the transfer efficiency of samples.

5.3.6. Immunohistochemistry

Uteri (Day 1–3 of the estrous cycle) were obtained from a slaughterhouse. Pieces of tissue were carefully excised and transferred to 10% neutral buffered formalin fixing solution for 24 h and then embedded in paraffin. Paraffin tissue sections (5 μm) were dewaxed, hydrated, and endogenous peroxidase activity quenched. They were then rinsed twice with PBS and subjected to immunohistochemical labeling with an avidin-biotin-peroxidase complex (ABC) method to examine MIF expression and secretion. All primary antibodies were labeled with the peroxidase Vectastain elite ABC Kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine tetrahydrochloride (DAB substrate kit; Vector Laboratories) was used as the substrate-chromogen solution. Negative controls were obtained by omitting primary antibodies. Bovine corpus luteum was used as positive control. After development of the immunoreaction, the slides were counterstained with hematoxylin.

5.3.7. Statistical Analysis

Each experiment was carried out using the cells from one uterus and was repeated with three different uteri. Northern and Western blots were run in triplicate for each uterus. For MIF expression, the data were analyzed by two-way ANOVA, which included the main effects of time and treatment (control, IFN-τ) and the time x

treatment interaction. Differences between individual means were determined by Tukey honestly significant difference test. A probability of $P < 0.05$ was considered to be statistically significant. The data were analyzed using the computer program JMP (SAS Institute Inc., Cary, NC).

5.4. Results

5.4.1. Effect of INF-τ on Secretion of Newly Synthesized Proteins from Endometrial Epithelial Cells

To determine the effect of IFN-τ on protein secretion, the confluent cultures of bovine endometrial epithelial cells were treated with or without IFN-τ and the ^{35}S-methionine labeled proteins were analyzed by 2D PAGE. Figure 1 shows representative 2D gel autoradiographs of labeled proteins from control and IFN-τ treated cells. A comparison of control and IFN-τ-treated cells showed that two protein spots, one with the estimated pI of 4.8 and molecular mass of 76 kDa (designated P76) and the other with a pI of 6.7 and molecular mass of 12 kDa (designated P12), were present in IFN-τ but not in control cells.

5.4.2. Identification of P12

In-gel digestion and sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nanoelectrospray tandem mass spectrometry on a Finnigan LCQDECA quadruple ion trap mass spectrometer. The peptide sequence (**LLCGLLTER**) that resulted in positive identification of P12 was located in the N-terminal part of bovine MIF (amino acid sequence of bovine MIF from Genbank Database [accession number AAB32021]): PMFVVNTNVP RASVPDGLLS ELTQQLAQAT GKPAQYIAVH

VVPDQLMTFG GSSEPCALCS LHSIGKIGGA QNRSYSKLLC GLLTERLRIS PDRIYINYYD MNAANVGWNG STFA.

5.4.3. Effect of IFN-τ on MIF mRNA and Protein Expression in Endometrial Cells

A single band at approximately 600 bp (the predicted size for the MIF mRNA) was observed in all epithelial RNA samples (Fig. 2). Densitometric analysis of the 600-bp bands, and normalization of these values to those of the respective GAPDH band, revealed that expression did not change with time of incubation and that there was no effect of IFN-τ on MIF mRNA levels at any of the time points. No band corresponding to MIF mRNA was observed in samples from stromal cells (data not shown).

To determine whether MIF protein was expressed in the endometrial cells, cellular extracts were analyzed by Western blotting using mouse anti-human MIF antibody. Results show a strong immunoreactive signal in all samples of epithelial cells. The amount of MIF did not change with time, and there was no effect of IFN-τ (Fig. 3). MIF protein was not detectable in the stromal cells (data not shown).

5.4.4. Analysis of MIF Protein Secretion from the Endometrial Epithelial Cells

To determine whether IFN-τ altered the secretion of MIF protein from the epithelial cells, proteins present in the culture medium were analyzed by Western blotting using mouse anti-human MIF antibody. The results showed a strong immunoreactive signal in the medium from bovine endometrial epithelial cells cultured in the presence of IFN-τ, compared with control samples (Fig. 4). In the

cells treated with IFN-τ, the amount of MIF secreted into the medium increased with time ($P < 0.01$) and was maximum at 12 h.

5.4.5.Expression of MIF in Bovine Endometrium

To determine whether MIF expression could be detected in vivo, immunohistochemistry was performed on formalin-fixed sections of bovine uteri taken at Days 1–3 of the cycle. Results showed MIF staining in the endometrium but not the myometrium. The staining was particularly intense on the apical side of the luminal epithelium and in the superficial glandular epithelium (Fig. 5, left image). No staining was observed in the epithelium of the deeper glands or in the subepithelial compact stroma.

5.5. Discussion

During early pregnancy, several changes take place in the reproductive tract in response to the presence of a viable conceptus. Previous studies have shown that IFN-τ stimulates the synthesis of a variety of proteins as well as preventing the luteolytic secretion of $PGF_{2\alpha}$. The present study has shown that IFN-τ induces the secretion of two newly synthesized proteins, one of which has been identified as MIF. This demonstrates for the first time that MIF is expressed in the ruminant endometrium in vivo. From the in vitro experiments, it appears that MIF is located specifically in the epithelial cells because it was not detected in the stromal cells in vitro. This agrees with the immunohistochemical data showing MIF in the luminal epithelium and the epithelium of the superficial glands. In our cell preparations, the epithelial cells are from luminal and superficial glands because they are obtained by enzymatic digestion of endometrial tissue from the side of the surface epithelium.

There do appear to be differences between epithelial cell types because no staining was observed in the epithelium of the deep glands. The stromal cells used for the in vitro studies are likely to be from the just under the surface epithelium and not from the deep stromal layer. Again, this agrees with the immunohistochemical data because no staining was observed in the subepithelial stroma, although MIF staining was observed in the deep stroma.

MIF was discovered as an activated T-lymphocyte-derived protein that inhibits the random migration of macrophages in vitro and is secreted by macrophages in response to cytokine stimulation. To date, its role as a proinflammatory cytokine involved in several aspects of the immune response has been demonstrated extensively [26]. Although there is information emerging concerning the presence of MIF in the uterus, its role in uterine function is not known. As in other animals, the endometrium of the cow contains immune cells [27]. Macrophages are widely distributed in female reproductive tissues and, in the endometrium, these cells represent an important mechanism of defense of its integrity and function. In the nonpregnant uterus, macrophage degradation of cellular debris and foreign material may be important in endometrial shedding and repair and in providing protection against infections.

It is now becoming clear, however, that MIF exerts a variety of biologic functions and MIF expression is found in cells other than activated T cells, indicating its involvement beyond the immune system in different pathophysiologic states [26, 28]. MIF mRNA is expressed in human ovary [29] and ovulated oocytes, zygotes, two-cell embryos, eight-cell embryos, and blastocysts of mice [30] which, together with other findings, suggests a possible role of MIF in different aspects of reproduction, such as ovulation, blastocyst implantation, and embryogenesis [31]. In

human pregnancy, a sarcolectin-binding protein whose properties corresponded to those of MIF has been described in term placenta [32]. Recent reports have shown that MIF is expressed in the human endometrium [33] and by first-trimester trophoblasts, in which it can play a role during implantation and early embryonic development [34]. MIF mRNA is also expressed in the mouse ovary, oviduct, and uterus during the preimplantation period and all stages of the estrous cycle [30]. There is, however, no previous report in the literature on the presence of MIF in bovine endometrium.

The other important finding of this study is that secretion of MIF from the endometrial epithelial cells is stimulated by IFN-τ. Although IFN-τ can alter prostaglandin secretion from stromal cells of the bovine endometrium [35–37], it had no effect on the expression of MIF in these cells. IFN-τ did not appear to stimulate either MIF mRNA or protein in the cells but did increase the secretion of MIF. A possible explanation for this is that either IFN-τ stimulates the synthesis of MIF protein, and this is preferentially secreted, or that IFN-τ stimulates only the secretion of stored MIF. The anterior pituitary cells and macrophages of mice contain a significant amount of preformed MIF within intracellular pools that can be rapidly released on stimulation. This is in contrast to other proinflammatory cytokines, such as interleukin-1ß and tumor necrosis factor-α, that require de novo mRNA generation and protein synthesis before secretion is observed. There is little information on the role of MIF in early pregnancy in any species, and the present results suggest that the bovine conceptus has the capacity for local modulation of the production of cytokines that, in turn, may sustain development and maintain pregnancy.

During pregnancy in mice, macrophages are present at the fetomaternal interface, suggesting an involvement in both the response to infection and the

immune interactions between fetal and maternal tissues [38]. Despite such important physiological functions, the mechanisms involved in recruiting, maintaining, and activating macrophages in the uterus are not fully defined. However, several studies emphasize the central role of cytokines in these processes. It has been shown that uterine cells are a potent source of cytokines with well-defined functions in promoting monocyte migration and activation, such as colony-stimulating factor (CSF)-1, granulocyte-macrophage-CSF, monocyte chemotactic protein-1, and regulated upon activation, normal T cell expressed, and secreted [38]. Macrophages are the main target of MIF, which acts on these cells by inhibiting migration and increasing their scavenger activity [39, 40]. Based on this study, it is speculated that MIF may be involved in the macrophage accumulation and activation in the bovine endometrium. The endometrium of pregnant and nonpregnant uterus is also populated by NK cells. It has been proposed that in the nonpregnant uterus, they could affect growth, differentiation, breakdown, and regeneration of the uterine mucosa. In addition, in the endometrium during early pregnancy, these cells could influence implantation by controlling trophoblast invasion of the decidua and downregulating the immune response to the semiallogenic fetus [41, 42]. Several studies have been directed toward comprehension of the mechanisms regulating the activity of NK cells in human endometrium. In this context, the observation that the cytolytic activity of uterine NK cells can be regulated by cytokines, acting either as stimulatory or suppressive factors, is relevant [43, 44]. A remarkable feature of MIF is its immunosuppressive activity, as demonstrated by the recent study of Apte et al. [45]. These authors showed that MIF is able to inhibit NK cell-mediated cytolysis of both neoplastic and normal target cells, indicating that this cytokine can contribute to preserving immune privilege. In this respect MIF is similar to uterine milk proteins,

which are also secreted by the endometrium of ruminants and inhibit NK cells [20]. This inhibitory action may be important because activated NK cells can lyse trophoblast cells [16]. Thus, there is the potential involvement of MIF in the control of macrophage and NK cell activity in the bovine endometrium, which could be important for normal embryo development.

It is also possible that MIF can directly affect the nonimmune cells of the endometrium. For example, MIF could affect the growth and differentiation of the endometrial tissue and/or the embryo. It has been shown that MIF can function as an autocrine mediator of growth factor-dependent extracellularly regulated kinase, mitogen-activated protein kinase activation, and cell cycle progression [46]. In this way MIF is able to regulate proliferative and oncogenic processes. In light of the expression of MIF in the bovine endometrial epithelial cells, and its stimulation by IFN-τ, further work is needed to examine the effects of MIF on endometrial and embryonic cell function.

In conclusion, this study shows MIF mRNA and protein are expressed in cultured bovine endometrial epithelial cells and that the secretion of MIF is stimulated in response to IFN-τ in vitro. MIF expression is observed in vivo, particularly on the apical surface of the luminal epithelium and superficial glands of the endometrium. Taken together, our results suggest that MIF is likely a factor contributing to the establishment of early pregnancy; however, the functional significance of MIF remains to be determined. Understanding the regulation of MIF secretion and its site of action in the reproductive tract will add significantly to our understanding of early embryo-uterine interactions.

5.6. Acknowledment

We thank Daniell Rannou for technical assistance.

5.7. References

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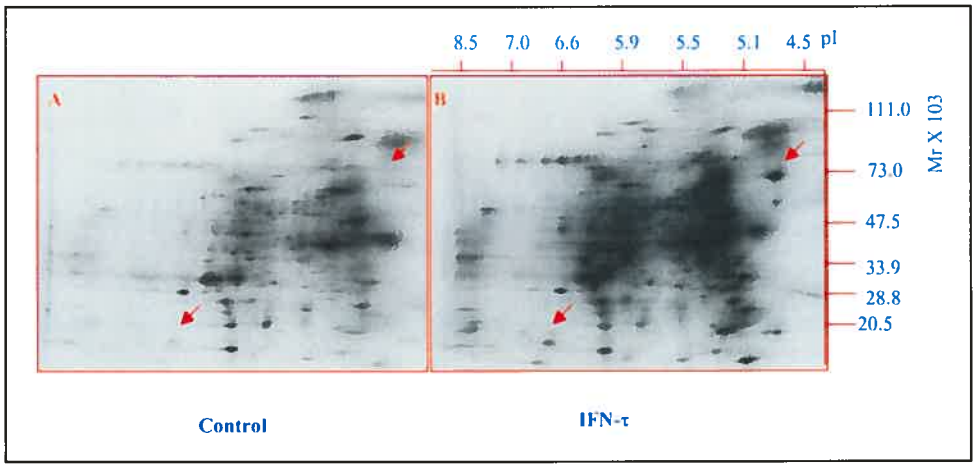

FIG. 1. Effect of IFN-τ on proteins secreted by endometrial epithelial cells. Cells were cultured with ^{35}S-methionine in the absence (**A**) or presence (**B**) of IFN-τ (100 ng/ml) for 24 h as described in *Materials and Methods.* Radiolabeled proteins in the medium were separated by 2D PAGE. The resulting representative autoradiographs were generated by exposure of x-ray film to the dried gels. Arrows show the position of protein spots upregulated by IFN-τ treatment. The analysis was repeated three times.

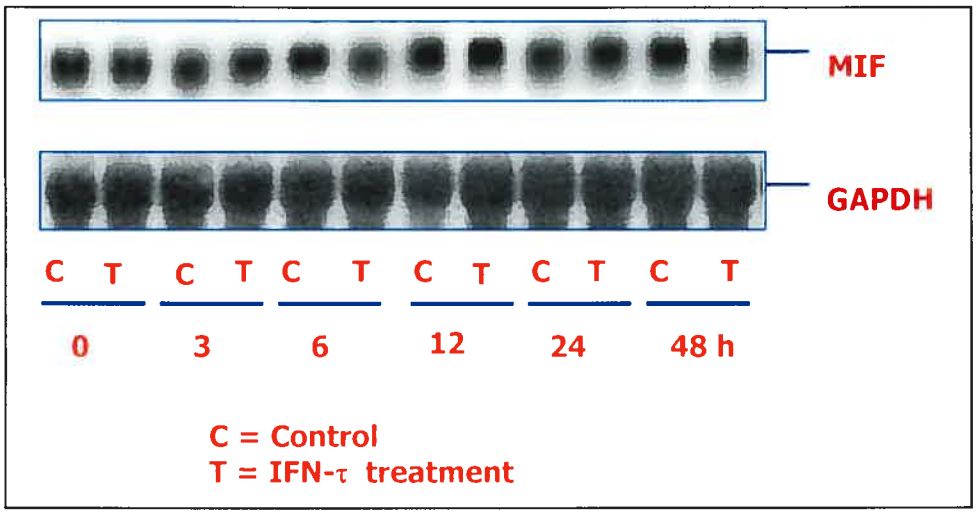

FIG. 2. Effect of IFN-τ on MIF mRNA expression in bovine endometrial epithelial cells. Cells were cultured to confluence and then incubated in the absence or presence of IFN-τ (100 ng/ml) for 0, 3, 6, 12, 24, 48 h. Total RNA was extracted from cells (three wells per time point) after treatment and samples (10 μg/lane) were analyzed by Northern blotting using ^{32}P-labeled MIF cDNA probe. The same blots were stripped of radioactivity and hybridized with a cDNA encoding the GAPDH as a control gene for RNA loading. A representative Northern blot from one experiment is shown; similar results were obtained from two other independent experiments. Markers on the right indicate migration of MIF mRNA (600 bp) and GAPDH mRNA (900 bp).

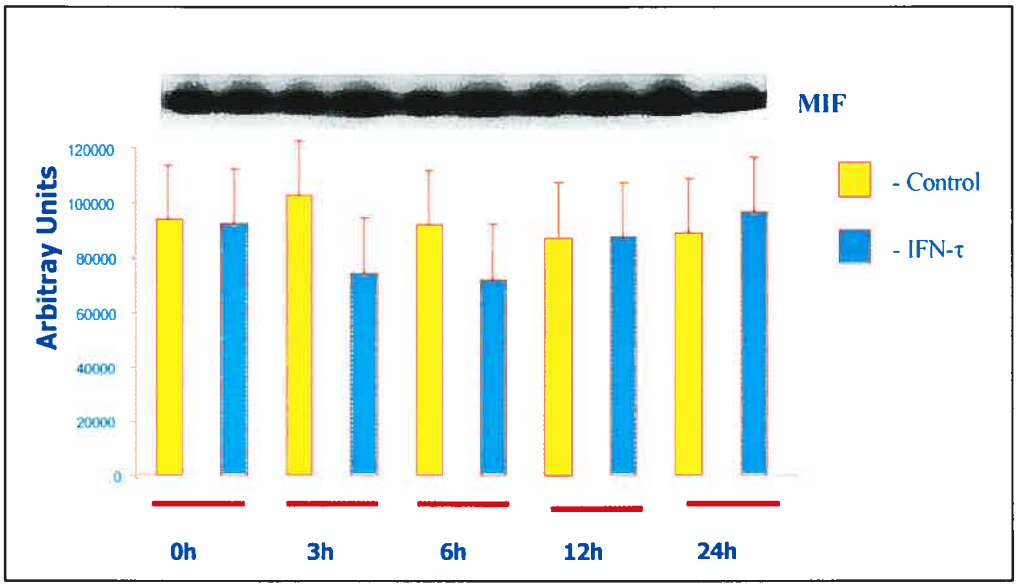

FIG. 3. Effect of IFN-τ on MIF protein expression in bovine endometrial epithelial cells. Cells were cultured to confluence and then incubated in the absence or presence of IFN-τ (100 ng/ml) for 0, 3, 6, 12, 24, 48 h. After treatment, the cells were harvested and cell proteins were extracted and quantified by Western blotting analysis (three wells per time point). A total of 25 μg protein per lane was loaded. Blots were scanned using a Storm PhosphorImager scanner and quantified as described in *Materials and Methods*. The upper band shows a representative blot, and the graph shows the quantitative measurement of band density from three independent experiments. Data are expressed as the least square means ± SEM (n = 3).

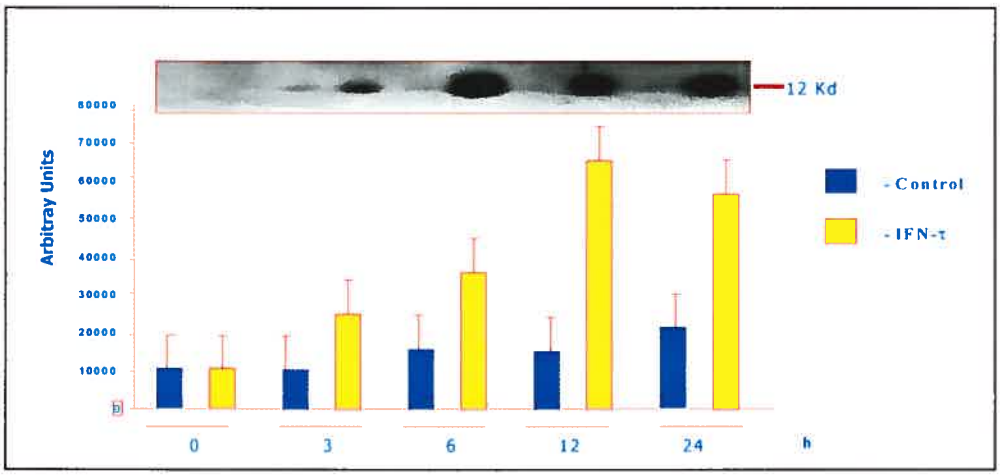

FIG. 4. Effect of IFN-τ on MIF secretion from bovine endometrial epithelial cells. Cells were cultured to confluence and then incubated in the absence or presence of IFN-τ (100 ng/ml) for 0, 3, 6, 12, 24, 48 h. After treatment, the culture medium (three wells per time point) was collected and proteins in medium were concentrated and quantified by Western blotting analysis. A total of 25 μg medium protein per lane was loaded. Blots were scanned using a Storm PhosphorImager scanner and quantified as described in *Materials and Methods*. The upper band shows a representative blot, and the graph shows the quantitative measurement of band density from three independent experiments. Data are expressed as the least square means ± SEM (n = 3)

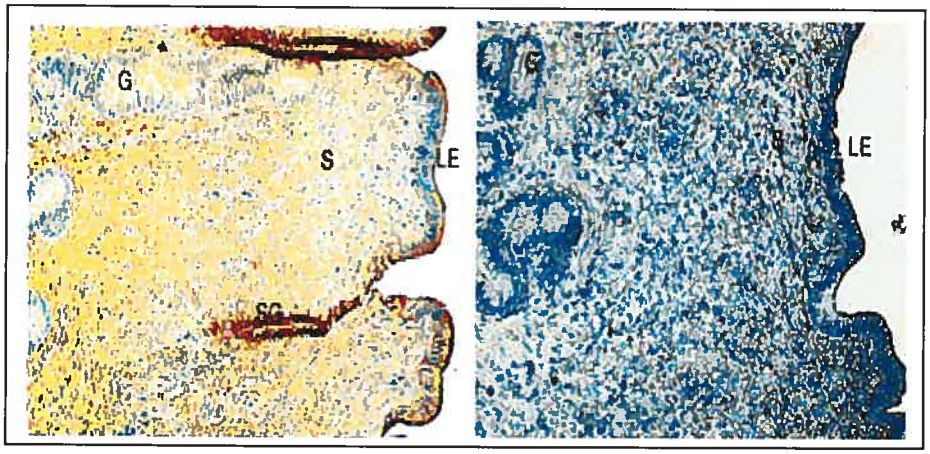

FIG. 5. Immunolocalization of MIF in bovine endometrium. Bovine uteri taken on Days 1–3 were subjected to immunohistochemical labeling with an ABC method to examine MIF expression and secretion. All primary antibodies were labeled with the Vectastain elite ABC Kit (Vector Laboratories). DAB substrate kit (Vector Laboratories) was used as the substrate-chromogen solution. Left image shows expression of MIF in bovine endometrium. Right image shows negative (without first antibody). After development of the immunoreaction, the slides were counterstained with hematoxylin. Magnification x200. LE, Luminal epithelium; S, stromal; G, gland; SG, superficial gland. The analysis was performed on four different tissue samples.

6. Article Three

Objective

To determine if IFN-τ induces apoptosis in epithelial cells of bovine endometrium.

BIOLOGY OF REPRODUCTION 68, 673–679 (2003)
Published online before print 27 November 2002.
DOI 10.1095/biolreprod.102.006924

Progesterone-Modulated Induction of Apoptosis by Interferon-Tau in Cultured Epithelial Cells of Bovine Endometrium[1]

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ABSTRACT

Interferon-tau (IFN-τ) is produced by the trophoblast prior to implantation in ruminants. It is involved in maternal recognition of pregnancy, and is a pleiotropic molecule that can alter the synthesis of endometrial proteins and inhibit proliferation of some cells. We have observed that IFN-τ reduces the DNA content in cultures of bovine endometrial epithelial cells; therefore, the objective of this study was to determine whether IFN-τ would induce apoptosis in bovine endometrial cells. Epithelial cells were prepared, cultured to confluence, and then incubated for 24 or 48 h in the presence or absence of 10 ng/ml progesterone, 100 ng/ml IFN-τ, or 10 μg/ml cycloheximide (CHX; an apoptosis inducer used as a positive control). Cells undergoing apoptosis exhibit such characteristics as the appearance of apoptotic bodies and DNA fragmentation. The incidence of apoptosis was assessed by using TUNEL, DNA fragmentation analysis, and Western blot analysis of Bax-α protein expression. The results showed that IFN-τ and CHX significantly increased the percentage of cells with apoptotic nuclei (33.6% and 44.8%, respectively) compared with controls (11.7%; $P < 0.05$). Progesterone treatment of the cells significantly inhibited the ability of IFN-τ to induce apoptosis (14.6%) compared with IFN-τ alone (33.6%; $P < 0.05$). DNA fragmentation analysis showed that INF-τ and CHX treatment resulted in an increase in the appearance of DNA laddering compared with that in untreated control cultures. Western blot analysis showed that IFN-τ and CHX treatment resulted in a greater expression of the proapoptotic protein Bax-α compared with that in control cultures. These data demonstrate that IFN-τ can induce apoptosis in bovine uterine epithelial cells and that this effect is modulated by progesterone. We speculate that IFN-τ might play a critical role in the remodeling of the endometrium around the time of implantation.

apoptosis, implantation, pregnancy, progesterone, uterus

INTRODUCTION

Interferons (IFNs) are a family of cytokines that are involved in the regulation of immune and inflammatory responses. Interferon-tau (IFN-τ) is secreted specifically by the conceptus in ruminants during early pregnancy [1, 2]. IFN-τ, originally named trophoblastin or trophoblast protein-1, is a type I interferon that is involved in the establishment of early pregnancy in ruminants. Analysis of the cDNA and amino acid sequence revealed 45% to 68% identity between IFN-τ and IFN-α [3]; IFN-τ competes with IFN-α for the type I IFN receptor and possesses potent antiviral, antiproliferative, and immunomodulatory activities [4]. IFN-τ is released by the bovine conceptus as early as Day 9 of pregnancy [2] and serves as the signal for maternal recognition of pregnancy in domestic ruminants [5] by attenuating the release of prostaglandin $F_{2\alpha}$ and rescuing the corpus luteum from regression [6]. It can also alter the synthesis of endometrial proteins [7, 8], enhance the activity of 2′, 5′-oligoadenylate synthetase in endometrial stromal and epithelial cells, induce an antiviral activity in target cells [9], and inhibit the proliferation of lymphocytes [10] and oviductal epithelial cells [11]. These antiproliferative effects have been suggested to slow the growth of the endometrium during the period when the conceptus is elongating to establish contact with a high percentage of the surface epithelium of the uterine lumen [4].

Cell death is classified into two distinct types; namely, necrosis and apoptosis. Apoptosis is a typical form of programmed cell death that eliminates unwanted cells in the development of the immune system, organ formation, and embryogenesis [4]. The characteristic features of apoptosis are the condensation and fragmentation of nuclear chromatin, accompanied by the compaction of cellular organelles, dilatation of the endoplasmic reticulum, and a marked reduction in cell volume, all of which distinguish it from necrosis [12]. The occurrence of apoptosis has been described in many reproductive tissues including the uterine epithelium [13]. The induction of apoptosis in tissue and cells by IFN-γ and IFN-α as evidenced by studies on DNA fragmentation levels and DNA nick-end labeling has been reported [14–16], and Kim et al. [17] have shown that IFN-τ induces apoptosis in sheep hepatocytes. However, the activation of apoptosis by IFN-τ in bovine endometrium remains to be elucidated.

We have observed that IFN-τ decreases the DNA content in cultures of bovine endometrial epithelial cells. This could be due to initiation of apoptosis or to a decrease in cell proliferation. We hypothesize that apoptosis in bovine endometrium epithelial cells could be induced by IFN-τ. The objective in the present study was to elucidate the effect of IFN-τ on the induction of apoptosis in bovine endometrial cells. Because progesterone (P_4) signaling regulates the function and development of the bovine endometrium, we have examined the possibility that it modulates the effect of IFN-τ.

MATERIALS AND METHODS

Chemicals and Reagents

Cell culture medium (RPMI 1640), Hanks buffered saline solution (HBSS; calcium-free and magnesium-free), newborn calf serum (NBCS),

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Received: 25 April 2002.
First decision: 17 May 2002.
Accepted: 14 September 2002.

ISSN: 0006-3363. http://www.biolreprod.org

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Key words: Bovine, uterus, endometrial cells, interferon-tau, apoptosis, progesterone

[1] This work was supported by grants from NSERC (AKG)

6.1. Abstract

Interferon-tau (IFN-τ) is produced by the trophoblast prior to implantation in ruminants. It is involved in maternal recognition of pregnancy, and is a pleiotropic molecule that can alter the synthesis of endometrial proteins and inhibit proliferation of some cells. We have observed that IFN-τ reduces the DNA content in cultures of bovine endometrial epithelial cells; therefore, the objective of this study was to determine whether IFN-τ would induce apoptosis in bovine endometrial cells. Epithelial cells were prepared, cultured to confluence, and then incubated for 24 or 48 h in the presence or absence of 10 ng/ml progesterone, 100 ng/ml IFN-τ, or 10 μg/ml cycloheximide (CHX; an apoptosis inducer used as a positive control). Cells undergoing apoptosis exhibit such characteristics as the appearance of apoptotic bodies and DNA fragmentation. The incidence of apoptosis was assessed by using TUNEL, DNA fragmentation analysis, and Western blot analysis of Bax-α protein expression. The results showed that IFN-τ and CHX significantly increased the percentage of cells with apoptotic nuclei (33.6% and 44.8%, respectively) compared with controls (11.7%; $P < 0.05$). Progesterone treatment of the cells significantly inhibited the ability of IFN-τ to induce apoptosis (14.6%) compared with IFN-τ alone (33.6%; $P < 0.05$). DNA fragmentation analysis showed that INF-τ and CHX treatment resulted in an increase in the appearance of DNA laddering compared with that in untreated control cultures. Western blot analysis showed that IFN-τ and CHX treatment resulted in a greater expression of the proapoptotic protein Bax-α compared with that in control cultures. These data demonstrate that IFN-τ can induce apoptosis in bovine uterine epithelial cells and that this effect is modulated by

progesterone. We speculate that IFN-τ might play a critical role in the remodeling of the endometrium around the time of implantation.

6.2. Introduction

Interferons (IFNs) are a family of cytokines that are involved in the regulation of immune and inflammatory responses. Interferon-tau (IFN-τ) is secreted specifically by the conceptus in ruminants during early pregnancy [1, 2]. IFN-τ, originally named trophoblastin or trophoblast protein-1, is a type I interferon that is involved in the establishment of early pregnancy in ruminants. Analysis of the cDNA and amino acid sequence revealed 45% to 68% identity between IFN-τ and IFN-α [3]; IFN-τ competes with IFN-α for the type I IFN receptor and possesses potent antiviral, antiproliferative, and immunomodulatory activities [4]. IFN-τ is released by the bovine conceptus as early as Day 9 of pregnancy [2] and serves as the signal for maternal recognition of pregnancy in domestic ruminants [5] by attenuating the release of prostaglandin $F_{2\alpha}$ and rescuing the corpus luteum from regression [6]. It can also alter the synthesis of endometrial proteins [7, 8], enhance the activity of 2', 5'-oligoadenylate synthetase in endometrial stromal and epithelial cells, induce an antiviral activity in target cells [9], and inhibit the proliferation of lymphocytes [10] and oviductal epithelial cells [11]. These antiproliferative effects have been suggested to slow the growth of the endometrium during the period when the conceptus is elongating to establish contact with a high percentage of the surface epithelium of the uterine lumen [4].

Cell death is classified into two distinct types; namely, necrosis and apoptosis. Apoptosis is a typical form of programmed cell death that eliminates unwanted cells in the development of the immune system, organ formation, and embryogenesis [4].

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We have observed that IFN- τ decreases the DNA content in cultures of bovine endometrial epithelial cells. This could be due to initiation of apoptosis or to a decrease in cell proliferation. We hypothesize that apoptosis in bovine endometrium epithelial cells could be induced by IFN- τ. The objective in the present study was to elucidate the effect of IFN- τ on the induction of apoptosis in bovine endometrial cells. Because progesterone (P_4) signaling regulates the function and development of the bovine endometrium, we have examined the possibility that it modulates the effect of IFN- τ.

6.3. MATERIALS AND METHODS

6.3.1. Chemicals and Reagents

Cell culture medium (RPMI 1640), Hanks buffered saline solution (HBSS; calcium-free and magnesium-free), newborn calf serum (NBCS), antibiotics, and trypan blue were purchased from Gibco (Grand Island, NY). Collagenase (type II), trypsin (type III, from bovine pancreas), DNase I (type I, from bovine pancreas), gentamicin, BSA and paraformaldehyde (PFA) were purchased from Sigma

Chemical Company (St. Louis, MO). A stock solution of P_4 was prepared by dissolving the steroid in ethanol. Matrigel was obtained from VWR Canlab (Montreal, PQ, Canada). Protein assay-dye reagent concentrate was obtained from Bio-Rad Laboratories (Mississauga, ON, Canada). The in situ detection kit for programmed cell death (Mebstain Apoptosis Kit Direct) was purchased from Medical & Biological Laboratories Company, Ltd. (Naka-ku Nagoya, Japan). The recombinant bovine IFN-τ was a generous gift from Dr. R. Michael Roberts, University of Missouri. The activity of IFN-τ is 10.9×10^7 IU/mg and we have previously shown that it has no detectable endotoxin activity that might induce apoptosis [18].

6.3.2. Preparation and Culture of Cells

The epithelial cells were prepared as previously described [19]. Uteri from cows at Days 1 to 3 of the estrous cycle (ovaries with a corpus hemorrhagicum) were collected at the slaughterhouse and transported on ice to the laboratory. Cells prepared from endometrium at this stage respond to IFN-τ in a physiological manner; that is, IFN-τ inhibits the oxytocin stimulation of prostaglandin $F_{2\alpha}$ secretion [20]. Briefly, the two horns of the uteri were placed in sterile HBSS containing 100 U penicillin, 100 μg streptomycin, and 0.25 $\mu g/ml^{-1}$ amphotocerin. the myometrial layers were dissected from the two horns and the horns were then everted to expose the epithelium. The everted horns were digested for 2 h in HBSS with 0.3% (w/v) trypsin at 37°C to obtain epithelial cells. At the end of incubation, 10% NBCS in HBSS was added to inhibit the trypsin, the cell suspension was centrifuged at 60 x *g* for 5 min, and the pellet was washed three more times with HBSS. For further purification, the epithelial cell pellet was suspended in 20 ml of RPMI-1640 medium

supplemented with 5% NBCS and 50 mg/ml^{-1} gentamicin, plated onto 100 x 20 mm Petri dishes (Nunclon, Grand Island, NY), and incubated at 37°C with 5% CO_2 and 95% air for 3 h. At the end of incubation, contaminating stromal cells adhered to the dish and the floating epithelial cells were collected. After cell counting and viability determination by trypan blue-exclusion, 2.5 x 10^6 viable cells per dish were plated onto Matrigel-coated 6-well culture plates (200 µl of 11% Matrigel was added to each well and dried overnight).

6.3.3. Cell Proliferation Experiment

All cells were cultured in phenol red-free RPMI-1640 medium containing 5% fetal calf serum (depleted of steroids by dextran-charcoal extraction) at 37°C in humidified air (5% CO_2). After the epithelial cells had reached about 60% confluence, they were cultured for 4 days in medium alone or in medium containing P_4 (50 ng/ml), IFN- τ (10 or 100 ng/ml), or P_4 plus IFN- τ. The medium was changed every 2 days.

6.3.4. Determination of DNA Content

At the end of the culture period, the medium was removed and the cells were rinsed twice with HBSS and detached with 1 mmol EDTA in HBSS and the use of a rubber scraper. Cells were pelleted by centrifugation at 1000 x *g* for 5 min, and 100 µl of 0.2% (w/v) SDS in ETN buffer (10 nmol EDTA, 10 mmol Tris-HCl, and 100 mmol NaCl pH 7.0) was added to the pellet. The pellet was sonicated 15 times using a Branson sonifier-450 (VWR Canlab) at 10% power. The DNA content in a 10 µl sonicated cell suspension was determined using the bisbenzimide fluorescent dye method [21]. Calf thymus DNA was used as the standard.

6.3.5. Measurement of Total Protein

Total protein was measured in 10 μl of sonicated cell suspension using the Bradford method (Bio-Rad Laboratories). BSA was used as the standard.

6.3.6. Apoptosis Experiments

After the cells had reached confluence (about 7 days) they were cultured for 24 or 48 h in the presence or absence of P_4 (10 ng/ml), cycloheximide (CHX; 10 μg/ml), and IFN- τ (100 ng/ml). At the end of culture, the medium was collected and centrifuged at 500 x *g* for 5 min at 4°C to collect the cells. The cells attached to the dish were harvested by adding either 0.25% trypsin for the TUNEL labeling and DNA fragmentation studies or TED sonification buffer (20 mM Tris, 50 mM EDTA, and 0.1 mM diethyldithiocarbamic acid pH 8.0) containing 32 mM octyl glucoside for the analysis of Bax protein expression. Cells harvested from the dish were pooled with the cells from the medium.

6.3.7. DNA 3' End-Labeling and Quantification of Apoptosis

Cells were washed several times with PBS containing 2% NBCS and 0.1% NaN_3, centrifuged at 500 x *g* for 5 min at 4°C, and resuspended in PBS at a concentration of 10^5 cells/ml. Fifty microliters of cell suspension were applied to ProbePlus slides (Fisher Scientific Co., Nepean, ON, Canada). The ProbePlus slides were air-dried for about 1 h and cells were then fixed at 4°C for 15 min with 4% PFA (in 0.1 mM NaH_2PO_4 pH 7.4). The cells were permeabilized with 0.5% Tween-20 after removing the PFA and then DNA nick end-labeled, counterstained, and mounted as described in the Mebstain Apoptosis Kit directions. Negative control

slides were prepared from cells taken from each treatment group using precisely the same procedures, except that water was added instead of the terminal deoxynucleotidyl transferase (TdT). Positive controls were prepared by treatment with DNase I (1 μg/ml) at 37°C for 1 h prior to end labeling. Using this method, apoptotic cells appear green.

The incidence of apoptosis was evaluated by examining stained cells under a fluorescent microscope (Nikon Eclipse E800, Nikon Canada, Mississauga, ON, Canada) using the green filter set with excitation/emission wavelengths at 475/535 nm. The number of apoptotic epithelial cells was expressed as a percentage of total cells counted. One thousand cells were counted in each of three randomly selected microscopic fields and the average of these fields was used as one data point per experiment.

6.3.8. Gel Electrophoretic Analysis of Internucleosomal DNA Fragmentation

To estimate the degree of internucleosomal DNA fragmentation, the cells were lysed following the instructions provided by the manufacturer (TACS DNA Laddering Kit, R&D Systems, Minneapolis, MN). Genomic DNA was isolated from harvested cells and analyzed for oligonucleosomal DNA fragmentation as a marker for apoptosis. The DNA samples (15 μg) were electrophoretically separated on 1% agarose gels in 1x TAE buffer (90 mM Tris-acetic acid and 1 mM EDTA pH 8.0) at 90 V for 30 min. A 100-base pair (bp) DNA ladder (5 μl) was run with each gel as a molecular weight standard. The gel was stained with ethidium bromide (1 μg/ml) and viewed with UV light, then photographs were taken using the Foto/Analyst (version 1.1) gel documentation system (Fotodyne, Hartland, WI).

6.3.9. Analysis of Bax Protein Expression

Cell extracts were prepared as previously described [22] with minor modifications. After treatment, the harvested cells were sonicated (8 sec/cycle; 3 cycles) in 250 µl of TED sonification buffer. The sonicates were centrifuged at 13 000 x *g* for 25 min at 47°C and supernatants were stored at -70°C until immunoblotting analysis.

Supernatant proteins (25 µg) were resolved by one-dimensional SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes (Hybond-ECL; Amersham Life Science, Inc., Buckinghamshire, U.K.). The blots were incubated for 18 h at 4°C in the presence of rabbit anti-mouse Bax polyclonal immunoglobulin G (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) diluted to 1:400. Specificity of the reaction was verified by replacing the Bax antiserum with normal rabbit serum. Blots were washed, incubated with secondary antibody, and exposed to chemiluminescent detection substrates as described [23, 24]. The membranes were then scanned using a Storm 840 PhosphorImager scanner (Molecular Dynamics Inc., Sunnyvale, CA) and quantified by densitometry using ImageQuant software (version 1.2, Molecular Dynamics).

The molecular weight of the immunoreactive bands was determined by comparing them with a ladder of biotinylated SDS-PAGE molecular weight standards (Bio-Rad Laboratories, Hercules, CA) applied to a lane in each gel. Prestained standards were also applied to gels to assess the transfer efficiency of samples.

6.3.10. Statistical Analysis

Each treatment was carried out in triplicate using the cells from one uterus and each experiment was repeated with three different uteri. The effect of treatments was evaluated by least-squares ANOVA. The data in the form of percentages were arcsine-transformed before analysis. Individual comparisons between means were made by the Tukey-Kramer test. A probability of $P < 0.05$ was considered to be statistically significant.

6.4. Results

6.4.1. Effect of IFN- τ on DNA and Protein Content of Endometrial Epithelial Cells

IFN-τ at the 100 ng dose, either alone or in the presence of P_4, significantly decreased the DNA content of epithelial cells ($P < 0.001$; Fig. 1A), indicating that a decrease in proliferation or an increase in cell death, or both, occurred. The lower dose of IFN- τ increased the total protein content of epithelial cells but there was no effect of the high dose of IFN- τ (Fig. 1B). Progesterone caused an increase in cell protein content but it did not alter the response to IFN- τ.

6.4.2. Apoptosis in Endometrial Epithelial Cells

Apoptotic cells and apoptotic bodies were evident in the cultured cells following routine histological staining. Signs of apoptosis included cells with nuclei containing marginated chromatin; cells with a single, small, densely stained nucleus; cells with multiple, densely stained nuclear fragments; and membrane-bound structures containing condensed chromatin or cytoplasm (apoptotic bodies), or both. Cells with the abovementioned apoptotic morphological features were found using

the TUNEL method to contain fragmented DNA (Fig. 2). Staining was located predominantly in the nucleus and nuclear fragments. In the TUNEL-positive control (i.e., treated with DNase; Fig. 2E), labeling was observed in all cells, however, in the TUNEL-negative control (no terminal deoxynucleotidyl transferase; Fig. 2F), no labeling was observed, indicating that the labeling procedure was specific.

To determine the effect of IFN-τ and P_4 on the percentage of cells with apoptotic nuclei, confluent bovine endometrial epithelial cells were treated with or without IFN- τ and CHX in the presence or absence of P_4 for 48 h. The cells were harvested and fixed, and the genomic DNA nick end was labeled with TdT. The results show that IFN-τ and cycloheximide significantly increased the percentage of cells with apoptotic nuclei (33.6% and 44.8%, respectively) compared with controls (11.7%; $P < 0.05$). Progesterone treatment alone did not affect the number of dead cells but it significantly inhibited the ability of IFN- τ to induce apoptosis (14.6%) when compared with IFN- τ alone (33.6%; $P < 0.05$; Fig. 3). Progesterone also decreased CHX-induced apoptosis.

To further confirm the effect of IFN- τ and P_4 on apoptosis in endometrial epithelial cells, genomic DNA was isolated and analyzed for oligonucleosomal DNA fragmentation. DNA fragmentation analysis showed that INF- τ and CHX treatment resulted in an increase in the appearance of DNA laddering compared with that in untreated control cultures (Fig. 4). Progesterone treatment of cells significantly decreased the effect of IFN-τ treatment but had no effect on the fragmentation induced by CHX.

6.4.3. Effect of IFN- τ and P_4 on Expression of Bax-α

To determine whether the proapoptotic protein Bax-α was expressed following IFN-τ treatment, proteins extracted from cells were resolved by one-dimensional SDS-PAGE and analyzed by Western blotting using a specific anti-Bax-α antibody. The results show that IFN-τ, and CHX treatment increased the expression of Bax-α compared with that of control cultures. Progesterone treatment alone had no effect on Bax-α, but it significantly decreased the ability of IFN-τ to stimulate the expression of Bax-α (Fig.5; $P < 0.05$). However, P_4 did not diminish the induction of Bax-α by CHX.

To define whether the effect of IFN-τ on expression of Bax-α is time-dependent, cellular extracts were prepared from confluent endometrial epithelial cell cultures treated with IFN-τ at different times (0, 3, 6, 12, and 24 h). Extracted proteins were resolved by one-dimensional SDS-PAGE and analyzed by Western blotting using specific anti-Bax-α antibody. The results show that IFN-τ markedly increased Bax-α protein expression at all time points (Fig. 6). IFN-τ induced Bax-α in cultured bovine endometrial epithelial cells as early as 3 h ($P < 0.001$) and Bax-α continued to increase with time (Fig. 6).

6.5. Discussion

In this study we have demonstrated that IFN-τ inhibits epithelial cell proliferation and induces apoptosis in cultured bovine endometrial epithelial cells. Although the involvement of IFN-τ in maternal recognition of pregnancy has been known for a number of years, this is the first report that this cytokine directly induces a Bcl-2 gene family member, Bax-α, and apoptosis in bovine endometrial epithelial cells. The other important finding is that the IFN-τ -induced apoptosis is inhibited by

progesterone, which suggests that plasma progesterone concentration during early pregnancy is important for modulating the effect of IFN- τ.

Interferons are cytokines that play a complex and central role in the resistance of mammalian hosts to pathogens [25]. Interferons are widely recognized for their antiviral and antiproliferative effects, and these properties are exploited through their clinical application in the therapy of viral infections or malignant diseases [26]. Recent studies have resulted in a greater appreciation of immunomodulatory responses elicited by IFNs that are distinct from their ability to interfere with cell cycle progression and viral replication [27]. One biological function of IFNs is their ability to manipulate the events that mediate programmed cell death and many studies show that IFNs initiate apoptosis in a variety of cell types [28]. A few studies also illustrate the potential of IFNs to act as negative regulators of programmed cell death but the underlying mechanism remains unidentified [29, 30].

Although Kim et al. [17] have shown that high doses of ovine IFN- τ induced hepatocyte apoptosis in sheep, there have been no reports of the effect of IFN- τ on apoptosis in endometrial cells. Internucleosomal DNA fragmentation has been considered to be one of the earliest characteristic events of apoptosis [31] and this was detected after IFN- τ treatment of endometrial epithelial cells in the present study. These results provide biochemical evidence that IFN-τ induced endometrial epithelial cell death by apoptosis. In addition to the detection of oligonucleosomes in extracted DNA, the occurrence of apoptosis may also be inferred from the characteristic morphological appearance of degenerating cells, together with the detection of DNA fragmentation in single cells using TUNEL [32]. The role of IFN-τ -induced apoptosis is currently speculative because nothing is known of apoptosis in ruminant endometrium. Apoptosis could be involved in embryo attachment to the

endometrium and in placentation. It is known that in primates and rodents, uterine epithelial cells undergo extensive apoptosis with obvious morphological degradation during the early stage of pregnancy [33, 34]. Placentation in ruminants differs from that in primates and rodents in that the embryo does not invade into the endometrium; instead, trophoblast invasiveness in ruminants is limited to fusion of migrating binucleate cells with uterine epithelium. Considerable tissue remodeling and angiogenesis occur, however, within the endometrium at implantation. Thus, IFN-τ -induced apoptosis of endometrial epithelial cells could contribute to the marked endometrial remodeling associated with early placentation. The factors involved in inducing apoptosis in primate and rodent endometrium have not yet been elucidated, however, embryos in these species have been shown to secrete IFN-γ [35]. Thus, embryonic IFN could be involved in endometrial apoptosis observed at implantation. Because no information is available at present on apoptosis in the endometrium of ruminants, further studies need to be performed in vivo to investigate this possibility.

Bcl-associated X protein (Bax) is a member of the Bcl-2 gene family and has extensive amino acid sequence homology with Bcl-2 protein [36]. It is known that overexpression of Bax protein induces apoptotic cell death, and the action of Bax appears to be neutralized when heterodimerized with Bcl-2 and some other members of the Bcl-2 protein family that function as suppressors of cell death [36]. It has been proposed that the ratio of Bax to Bcl-2 and other antiapoptotic Bcl-2 family proteins appears to predetermine the life or death response of a cell to an apoptotic stimulus [36]. In the present study we sought to determine the expression of Bax-α protein in bovine endometrial epithelial cells and to evaluate Bax expression in relationship to DNA fragmentation and the apoptotic phenotype. The present results demonstrate that immunoreactivity for Bax-α increases after IFN-τ treatment of the cells in a

time-dependent manner. Thus it appears that IFN- τ -induced apoptosis is mediated by increased intracellular levels of Bax-α.

The corpus luteum is a transient endocrine organ that synthesizes P_4 to act on the uterus and support the developing embryo. In vivo, P_4 acts on an estradiol-primed uterus to stimulate growth of the endometrium and it is believed that cell death may be as important as cell proliferation in the regulation of normal uterine epithelial growth. There is mounting evidence to suggest that P_4 can inhibit apoptosis in a variety of P_4 receptor-positive tissues [37–41]. Nawaz et al. [42] reported that treatment of rabbits with P_4 dramatically decreased cell death in uterine epithelial cells. Results of the present study provide evidence of P_4 modulation of IFN-τ-induced apoptosis in bovine endometrial epithelial cells in vitro. The physiological significance of this remains to be established. Because IFN-τ is secreted from early blastocyst stage embryos and IFNs in general induce apoptosis, the role of P_4 could simply be to prevent IFN- τ -induced apoptotis, especially before the time of placentation. If IFN- τ -induced apoptosis is indeed involved in placentation, then it is possible that local P_4/IFN- τ concentrations at the sites of attachment determine whether there is an effect on the endometrium. Progesterone has been shown to alter the apoptotic threshold of endometrial cells in rats [43]. This suggests that production of IFN- τ by the embryo and P_4 by corpus luteum might be important for the establishment of pregnancy in addition to their role in preventing the secretion of the uterine luteolysin. Alterations in endometrial apoptosis, due to abnormal production of P_4, IFN- τ, or both may therefore represent an alternative cause of early pregnancy failure in cows.

In summary, our results demonstrate that IFN- τ significantly increased the percentage of endometrial epithelial cells with apoptotic nuclei, the appearance of

DNA laddering, and the expression of preapoptotic protein Bax-α. An interesting observation was that treating cells with P_4 significantly inhibited the ability of IFN-τ to induce apoptosis. These findings support the need for a balanced apoptosis regulatory process during the establishment of pregnancy. We speculate that the action of IFN-τ in bovine uterine epithelial cells plays a critical role in implantation and placentation during early pregnancy and that abnormal P_4, IFN-τ, or both could result in pregnancy failure for reasons other than failure to maintain the corpus luteum.

6.6. Acknowledgements

We thank Dr R. M. Roberts for the rbIFN-τ and Dr B. D. Murphy's group for their assistance.

6.7. REFERENCES

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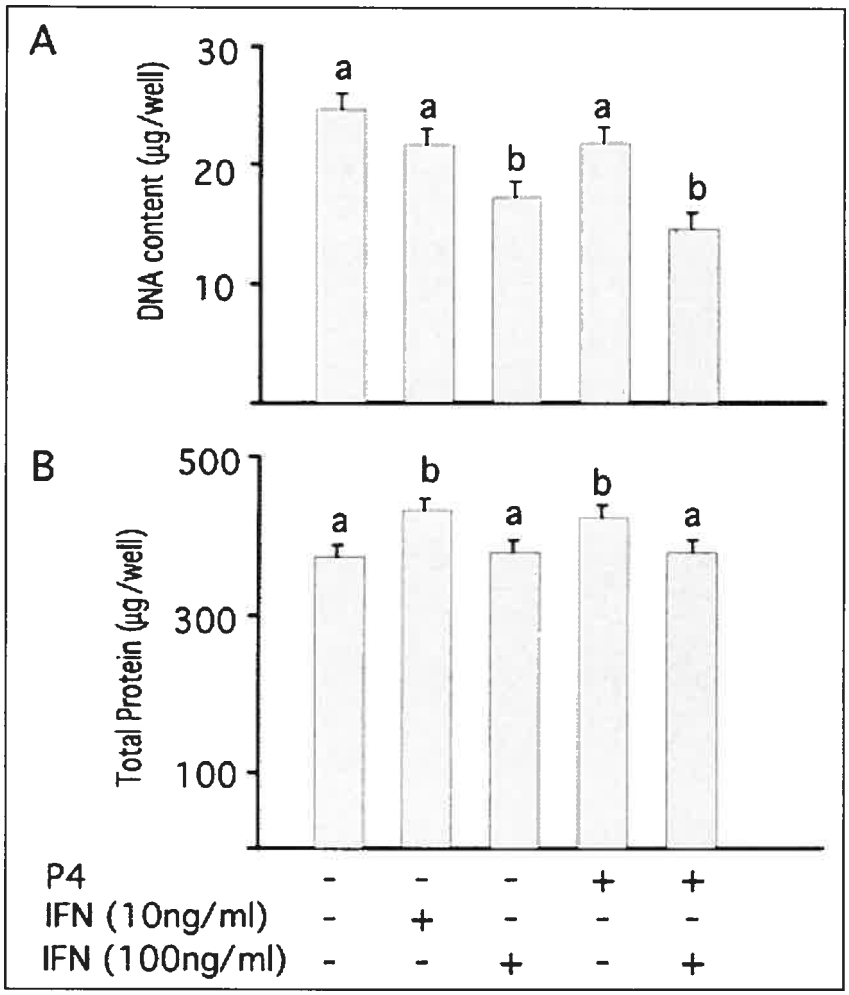

Fig. 1. Effects of P_4 and IFN-τ on **A**) DNA and **B**) protein content in bovine endometrial epithelial cells. Primary bovine endometrial epithelial cells were cultured with RPMI medium supplemented with 5% steroid-free fetal calf serum in the absence or presence of P_4 (50 ng/ml), IFN-τ (10 or 100 ng/ml), and P_4 + IFN-τ for 4 days. Data represent least-square means ± SEM. Bars with different letters are significantly different ($P < 0.05$).

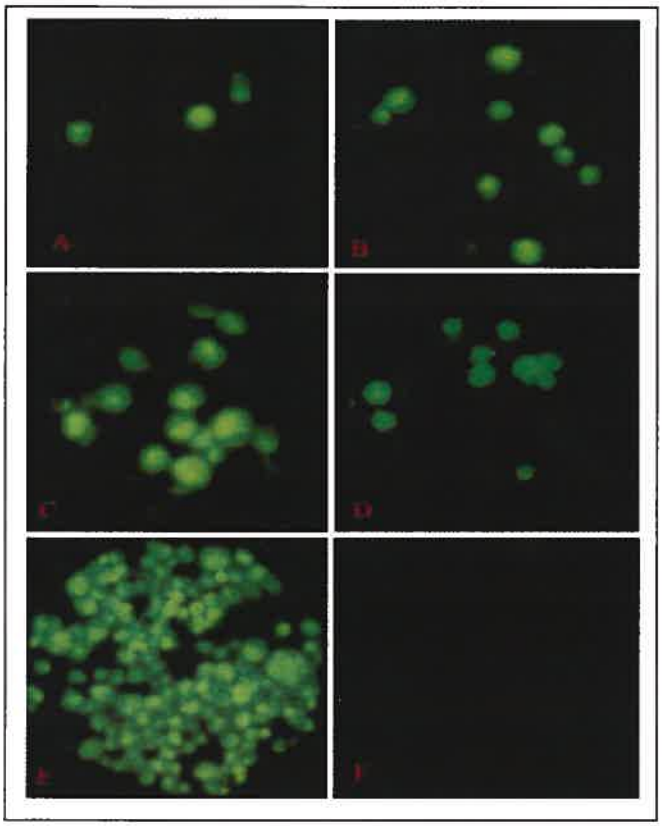

Fig. 2. Representative images of endometrial epithelial cells after TUNEL staining. Cells were cultured to confluence and then treated for 48 h with or without IFN-τ (100 ng/ml) or CHX (10 μg/ml). The cells were fixed and the genomic DNA nick end was labeled with deoxyuridine triphosphate (TUNEL) as described in the text. Apoptotic cells were observed with a fluorescence microscope and representative microscope fields are shown: **A**) cells cultured in medium alone (experimental control), **B**) IFN-τ, **C**) CHX, **D**) IFN-τ + CHX, **E**) positive control for TUNEL, and **F**) negative control for TUNEL. Each field had approximately the same umber of cells: 33, 30, 29, and 28 total cells for **A**, **B**, **C**, and **D**, respectively.

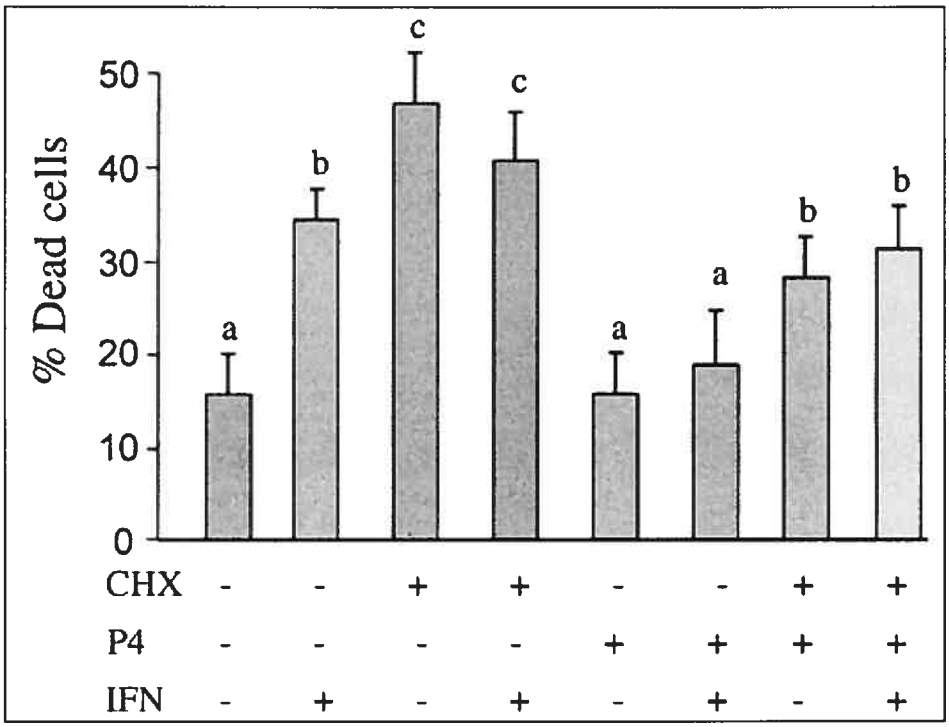

Fig. 3. The effect of IFN-τ and P_4 on the percentage of dead cells in primary culture of bovine endometrial epithelial cells. Isolated bovine endometrial epithelial cells were cultured to confluence before treatment for 48 h. The cells were fixed and the genomic DNA nick end was labeled with TdT as described in the text. The figure represents the quantitative measurement of apoptotic cells as a percentage of total cells counted. Groups with different letters are significantly different at the $P < 0.05$ level (n = 3). Data are expressed as the least-square means ± SEM (n = 3).

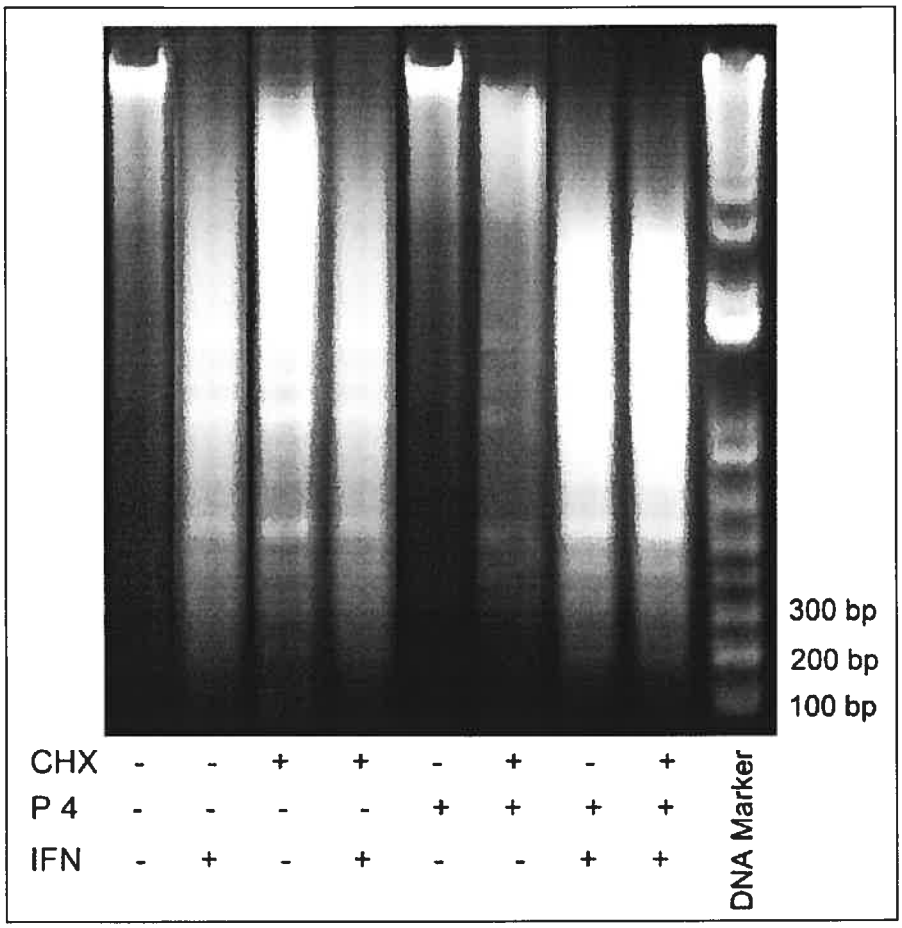

FIG. 4. The effect of 10 ng/ml P_4 on the onset of IFN-τ-induced apoptosis. Isolated bovine endometrial epithelial cells were cultured to confluence before treatment for 48 h. Genomic DNA was isolated from harvested cells and analyzed for oligonucleosomal DNA fragmentation by 1% agarose gel electrophoresis as a marker for apoptosis.

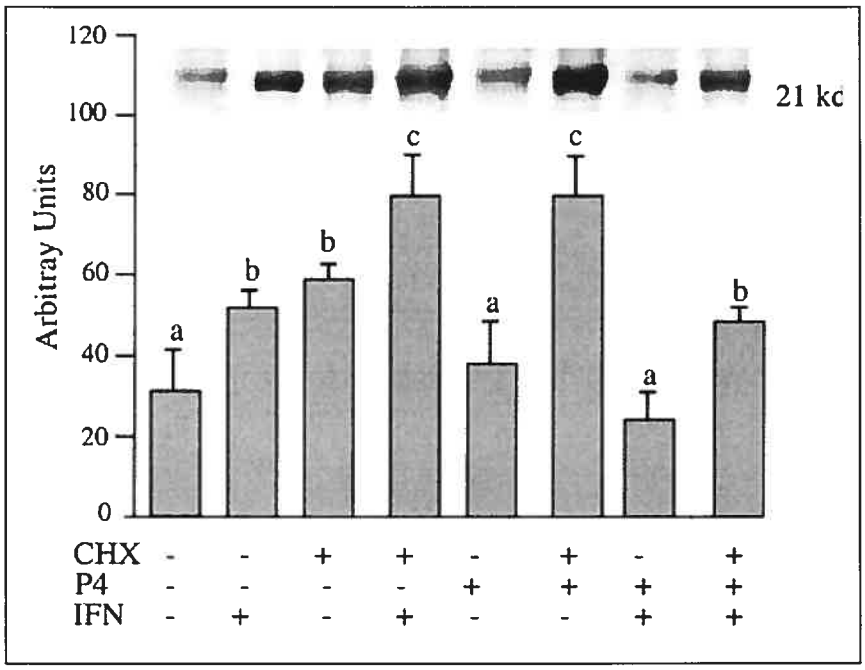

Fig. 5. Western blot analysis of proapoptotic protein Bax-α in epithelial cells. Isolated bovine endometrial epithelial cells were cultured to confluence before treatment. After treatment for 48 h, the cells were harvested and cell proteins were extracted. Protein (25 µg per lane) was loaded and the membranes were incubated with mouse Bax-α antibody, and enhanced chemiluminescence was used to visualize immunopositive protein. Blots were scanned with a Storm PhosphorImager scanner and quantitated as described in *Materials and Methods.* The upper band shows a representative blot and the graph shows the quantitative measurement of band density of control, CHX, and IFN-τ treatment in the presence and absence of P_4. Data are expressed as the least-square means ± SEM (n = 3). The letters represent significance at the $P < 0.05$ level.

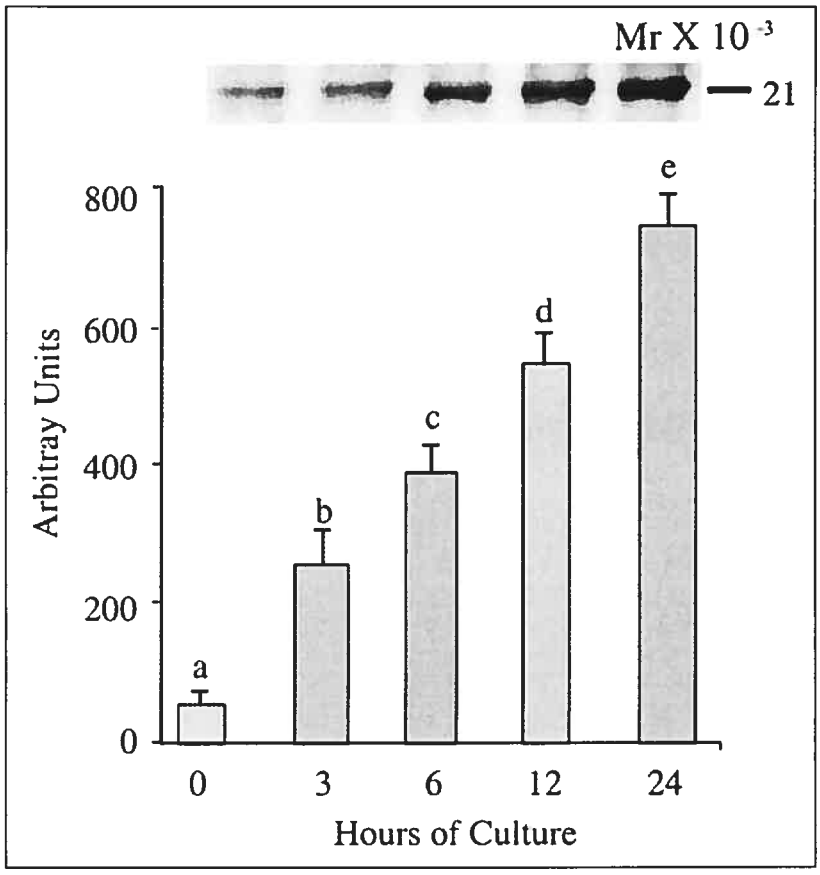

Fig. 6. Effect of IFN-τ on expression of proapoptotic protein Bax-α in epithelial cells. Isolated bovine endometrial epithelial cells were cultured to confluence then incubated in serum-free RPMI medium in presence or absence of 100 ng/ml IFN-τ for 0, 3, 6, 12, or 24 h. At the end of incubation, cells were harvested and cell proteins were extracted. Protein (25 µg per lane) was loaded and the membranes were incubated with mouse Bax-α antibody, and enhanced chemiluminescence was used to visualize immunopositive protein. Blots were scanned using a Storm PhosphorImager and quantitated as described in *Materials and Methods*. The upper band shows a representative blot and the graph shows the quantitative measurement of band density of control and IFN-τ treated cells. Data are expressed as the least-square means ± SEM (n = 3). The letters represent significance at the $P < 0.05$ level.

7. General Discussion

My project provides novel information on the effect of IFN-τ on protein secretion in bovine endometrial cells and its modulation by steroid hormones. These will be generally discussed in the following three aspects.

7.1. Proliferation and apoptosis of bovine endometrial epithelial cell and the establishment of early pregnancy

In the first study we have demonstrated that IFN-τ inhibits epithelial cell proliferation and induces apoptosis in cultured bovine endometrial epithelial cells. Previous reports have shown that the regulation of apoptosis is critical throughout pregnancy encompassing both the growth and remodelling phases of the placenta. During the growth phase, the balance between cell death by apoptosis and proliferation is a key determinant of pregnancy success (Lea, Riley et al. 1999). One biological function of IFNs is their ability to manipulate the events that mediate programmed cell death. Many studies show that IFNs initiate apoptosis in a variety of cell types (Otsuki, Yamada et al. 1998). A few studies also illustrate the potential of IFNs to act as negative regulators of programmed cell death but the underlying mechanism remains unidentified (Burgering and Coffer 1995; Egle, Villunger et al. 1996). Although Kim et al. (Kim, Stoica et al. 2000) have shown that high doses of ovine IFN-τ induced hepatocyte apoptosis in sheep, there have been no reports of the effect of IFN-τ on apoptosis in endometrial cells. Results from my first study demonstrates that INF-τ at the 100 ng dose significantly inhibits epithelial cell proliferation and

induces apoptosis in cultured bovine endometrial epithelial cells. DNA 3' end-labelling and apoptosis quantification analysis revealed that INF-τ significantly increased the percentage of cells with apoptotic nuclei compared with controls. It has been recently demonstrated that apoptosis in the endometrial glands can serve as a marker for receptive endometrium at implantation. von Rango et al. detected apoptosis in the glandular epithelium of the basalis at the beginning of the implantation window that extended to the functional is in the luteal phase (von Rango, Classen-Linke et al. 1998). Proliferation and bcl-2 expression, which are predominant in the glandular compartment during the proliferative phase, are limited to the stromal compartment during the luteal phase of the menstrual cycle (von Rango, Classen-Linke et al. 1998; Yamashita, Otsuki et al. 1999) . Moreover, apoptosis may be related to the loss of the protective effect of bcl-2 which is accompanied by increased expression of bax protein (Akcali, Khan et al. 1996; von Rango, Classen-Linke et al. 1998). Apoptotic DNA-fragmentation has been demonstrated in CTBs, being most abundant in early pregnancy (Quenby, Brazeau et al. 1998). In contrast, bcl-2 protein expression has been found in STBs, being less abundant in early pregnancy (Lea, al-Sharekh et al. 1997; Mochizuki, Maruo et al. 1998). These data indicate that early placenta is characterised by a highly proliferative activity of CTB cells associated with increased occurrence of apoptosis (Mochizuki, Maruo et al. 1998). Therefore, bcl-2 may prevent apoptosis in STB (Quenby, Brazeau et al. 1998; Toki, Horiuchi et al. 1999). In contrast to normal pregnancy, apoptotic cells are predominant in the STB layer in cases of spontaneous abortion

(Kokawa, Shikone et al. 1999). Placental apoptosis increases as pregnancy progresses which suggest that it is a normal physiological phenomenon throughout gestation (Smith, Baker et al. 1997). TRAIL, like FasL, seems to provide regulation of placental homeostasis during trophoblast invasion. These findings provide a potential explanation for villous remodelling during placentogenesis (Uckan, Steele et al. 1997; Phillips, Ni et al. 1999).

Internucleosomal DNA fragmentation was detected after IFN-τ treatment of endometrial epithelial cells in the present study. These results provide biochemical evidence that IFN-τ induced endometrial epithelial cell death by apoptosis. In addition to the detection of oligonucleosomes in extracted DNA, the occurrence of apoptosis may also be inferred from the characteristic morphological appearance of degenerating cells, together with the detection of DNA fragmentation in single cells using TUNEL (Palumbo and Yeh 1994). These results are supported by observations in the rhesus monkey during early stage of pregnancy. In situ 3'-end-labeling results showed that glandular epithelial cells underwent extensive apoptosis with obvious morphological degradation during the early stage of pregnancy (Gao, Fu et al. 2001).

7.2. Biological function of MIF protein and the establishment of early pregnancy

Results from the second study show that MIF mRNA and protein were present in the epithelial cells but not the stromal cells. There was no effect of IFN-τ on MIF expression in the epithelial cells. However, IFN-τ significantly

stimulated the secretion of MIF protein from the cells. These findings indicate the MIF protein has a potential role in bovine uterine during the establishment of early pregnancy. MIF differs from most other cytokines with respect to its expression profile. Cytokines are usually produced upon induction. In contrast, MIF is constitutively expressed in a variety of immune and non-immune cells and its tissue distribution is almost ubiquitous. It has therefore been suggested that release of MIF into the circulation occurs from preformed intracellular stores (Benigni, Atsumi et al. 2000).

The possible role of MIF in the establishment of early pregnancy and in the cytokine network for oocyte development has been reported (Wada, Fujimoto et al. 1997). Cytokines are the major mediators of pathophysiologic events in reproductive processes and exert profound effects on ovarian function. MIF mRNA is expressed in ovulated oocytes, zygotes, two-cell embryos, eight-cell embryos, and blastocysts of mice (Suzuki, Kanagawa et al. 1996). In addition, a high level of MIF mRNA expression is found in osteoblasts (Onodera, Suzuki et al. 1996). These findings indicate the involvement of MIF in cell growth during the early stages of organogenesis. *In vitro*, the MIF mRNA is expressed in a mouse osteoblastic cell line, MC3T3-E1 and it has been demonstrated that TGF-β, bFGF, insulin-like growth factor-2 (IGF-2), bovine and fetal serum (FBS) markedly upregulate MIF mRNA expression (Onodera, Suzuki et al. 1999) (Figure 1). This suggests that MIF could be involved in embryogenesis, cell growth and differentiation.

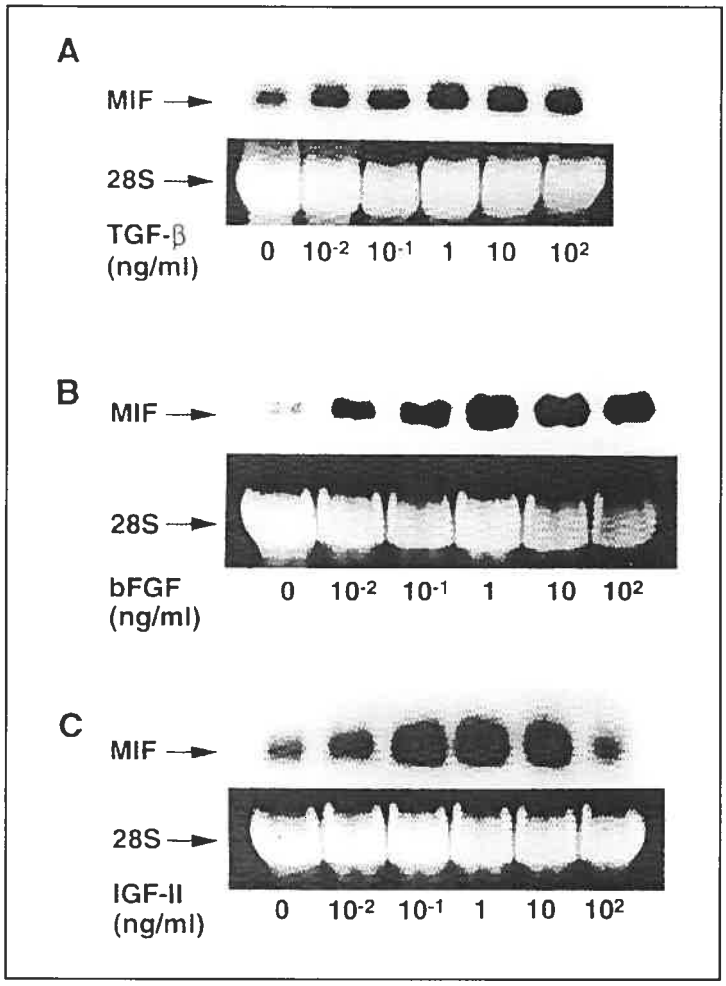

Figure 1. Effects of growth factors on the expression of MIF mRNA in MC3T3-E1 cells. Total RNA of cells from subconfluent cultures stimulated with various concentrations of growth factors were subjected to Northern blot analysis and hybridized with a [^{32}P] –labeled rat MIF cDNA. MIF transcripts of around 0.6 kb visualized by autoradiography are shown, and 28S ribosomal RNA bands stained with ethidium bromide are shown at the bottom of each lane. (A) TGF-β. (B) bFGF. (C) IGF-II (Adopted from Onodera, Suzuki et al. 1999)

It was reported that human villous tissue contains a significant amount of MIF in the cytotrophoblasts of both the inner layer of villi and the trophoblastic cell islands (Arcuri, Cintorino et al. 1999). It is therefore, thought that MIF might play a critical role not only in early embryonic development but also in implantation. Some scientists demonstrated that the MIF expression pattern parallels tissue specification and organogenesis during embryogenesis (Kobayashi, Satomura et al. 1999). Thus, it is likely that MIF plays an important role throughout developmental processes of early pregnancy.

7.3. Interactions of IFN-τ, E2 and P4 and the establishment of early pregnancy

The present study is the first to simultaneously examine the effects of embryo pieces, IFN-τ and embryo conditioned medium on protein secretion from bovine endometrial epithelial cells and the hormone modulation of IFN-τ induced protein secretion. The current study demonstrates that embryo pieces, IFN-τ induced P12 and P76 protein secretion in cultured bovine endometrial epithelial cells. The secretion level of these two protein sports are up-regulated by P4 and down-regulated by E2. Pregnancy is established and maintained in ruminants in response to interactions between the conceptus (embryo and associated membranes), uterus and or ovarian corpus luteum (CL). These interactions prevent functional and structural regression of the CL, or luteolysis, in response to episodic release of the luteolytic hormone prostaglandin F 2α (PGF) from the uterine endometrium. The functional life-span of CL of ruminants is extended by pregnancy recognition signals from the trophoblast

which act in a paracrine manner to abrogate the mechanism responsible for the pulsatile release of PGF required for luteolysis. The signal for pregnancy recognition in ruminants is IFN-τ. The expression of IFN-τ is regulated developmentally to the period of pregnancy. Previous report demonstrated that a protein [ovine trophoblast protein 1 (oTP-1)] secreted by the pre-implantation blastcyst binds specifically to ovine endometrium and increases the rate of protein release by endometrial explants from nonpregnant ewes on day 12. Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-PAGE) revealed that the synthesis of six polypeptides was selectively stimulated in these explants by the presence of oTP-1. Subsequent extension of their study indicated that oTP-1 also decreased secretion of some polypeptides by such cultures (Godkin, Bazer et al. 1984). The present data, together with previous findings, suggest that increased and/or decreased concentrations of some proteins may be involved in regulating steroid-homone-related uterine adaptations during pregnancy.

P4 is the hormone of pregnancy and unequivocally required in all mammals for maternal support of conceptus (embryo/fetus and associated membrances) survival and development. The action of P4 are mediated by the progesterone receptor (PR). The corpus luteum is a transient endocrine organ that synthesizes P_4 to act on the uterus and support the developing embryo. This suggests that production of IFN-τ by the embryo and P_4 by corpus luteum might be important for the establishment of pregnancy in addition to their role in preventing the secretion of the uterine luteolysin.

It is well documented that circulatory progesterone levels are increased during pregnancy and decrease at term. Our study suggest that P4 up-regulates some protein secretion in emdometrium and, therefore, maintains uterine adaptions for embryo implantation during pregnancy, when P4 levels are elevated. Our results show that both of P12 and P76 secretion induced by IFN-τ were elevated with P4 treatment, whereas decreased with E2 treatment. Several studies suggest a role for estrogens in the regulation of protein expression and secretion in human umbilical vein endothelial cells, 17 beta-E2 transiently down-regulates the expression and secretion of a potent negative regulator of angiogenesis, thrombospondin-1 (Sengupta, Banerjee et al. 2004). Interestingly, in the present study, we observed a signigicant deciline in P12 and P76 protein secretion in bovine endometrial epithelial cells treated with E2. The IFN-τ-induced changes in P12 and P76 protein and its modulation by steroid hormone may potentially affect the mechanisms associated with maintenance of pregnancy.

In summary, this project demonstrated that embryonic IFN-τ can induce protein secretion from cultured bovine endometrial epithelial cells, stimulate MIF secretion and induce apoptosis in bovine endometrial epithelial cells. These effects were modulated by steroid hormones. The most significant findings in those studies are that: (1) it demonstrates for the first time that MIF mRNA and protein are highly expressed in cultured bovine endometrial epithelial cells and that the secretion of MIF is stimulated in response to IFN-τ. (2) IFN-τ inhibits

epithelial cell proliferation and induces apoptosis in cultured bovine endometrial epithelial cells. Although the involvement of IFN-τ in maternal recognition of pregnancy has been known for a number of years, this is the first report that this cytokine directly induces a bcl2 gene family member, Bax-α, and apoptosis in bovine endometrial epithelial cells. The other important finding is that the IFN-τ-induced apoptosis is inhibited by progesterone, which suggests that plasma progesterone concentration during early pregnancy is important for modulating the effect of IFN-τ. Taken together, our results suggest that MIF is likely a factor contributing to the establishment of early pregnancy, however, the functional significance of MIF remains to be determined. Additional studies are required to further assess the mechanisms responsible for progesterone-mediated apoptosis during pregnancy. Understanding the effect of IFN-τ on protein secretion and its modulation by steroid hormones in the reproductive tract will add significantly to our understanding of early embryo-uterine interactions.

8. General Conclusions

The objective of this work was to examine the effect of IFN-τ on protein secretion and the modulation of its effects by steroid hormones, in order to establish the mechanisms involved in the establishment of early pregnancy. In this project, three major conclusions are as followings:

(1). IFN-τ significantly increased the percentage of endomentrial cells with apoptotic nuclei, the appearance of DNA laddering, and the expression of pre-apoptotic protein Bax-a. Treating cells with P4 significantly inhibited the ability of IFN-τ to induce apoptosis. These findings support the need for a balanced apoptosis regulatory process during the establishment of pregnancy.

(2). IFN-τ induced the secretion of two major protein spots (P12 and P76) from bovine uterine epithelial cells and this was modulated by steroid hormones. E2 down-regulated and P4 up-regulated protein secretion induced by IFN-τ. Protein sequence analysis of proteins (P12) induced by IFN-τ shown partial amino acid sequences that corresponded to MIF. These findings indicated that E2, P4 and IFN-τ have differential effects in the regulation of uterine protein secretion.

(3). MIF mRNA and protein are expressed in cultured bovine endometrial epithelial cells, but not in stromal cells. There was no effect of IFN-τ on MIF expression in the epithelial cells. However, the secretion of MIF from bovine endometrial epithelial cells is stimulated in response to IFN-τ in

vitro. These findings suggest that MIF is potentially involved in the establishment of early pregnancy.

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Wang, Bingtuan		[redacted]
Sigle du programme	**Titre du programme**	**Option**
M.Sc. Ph.D.	Sciences vétérinaires	Reproduction

DESCRIPTION DE L'ARTICLE

Auteurs
Wang, Bingtuan and Alan K. Goff
Titre
Influence of estrodial and progesterone on protein secretion induced by recombinant bovine interferon-tau in bovine endometrial cells

Revue	**Date de publication**
Reproduction	in preparation

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DESCRIPTION DE L'ARTICLE

Auteurs	
Wang, Bingtuan; Xiao, Chaowu; Goff, Alan K.	
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Progesterone-modulated induction of apoptosis by interferon-tau in cultured epithelial cells of bovine endometrium	
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Auteurs	
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Titre	
Interferon-tau stimulates secretion of macrophage migration inhititory factory from bovine endometrial epithelial cells	
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Auteurs
Wang B. Xiao C, Goff AK

Titre
Progesterone-modulated induction of apoptosis by interferon-tau in cultured epithelial cells of bovine endometrium.

Numéro de la revue	Pages	Date
68	673-679	2003

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Ph.D.	Sciences vétérinaires	Reproduction

DESCRIPTION DE L'ARTICLE

Auteurs
Wang B, Goff AK.
Titre
Interferon-tau stimulates secretion of macrophage migration inhibitory factor from bovine endometrial epithelial cells.

Numéro de la revue	Pages	Date
69	1690-1696	2003

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